Shellfish Microbiology: A literature review including conclusions and recommendations to address current and future research needs.

A report prepared for the Shellfish Enhancement Task Force
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Council on the Environment Commonwealth of Virginia

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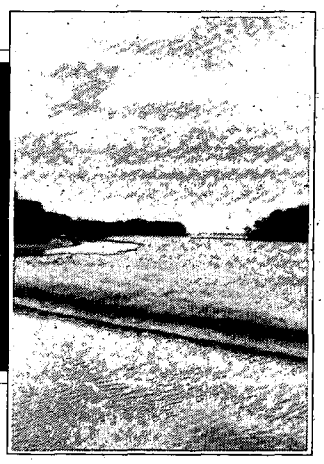

ı Coastal Resources Management Program links state programs to manage coastal resources. n's coastal boundary includes the 29 counties and 15 cities within Tidewater Virginia. The coordinated and monitored by the Virginia Council on the Environment.

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Highlights and Summary of Literature Review:

1. The coliform and fecal coliform indicators were chosen as indicators of sanitary water quality on the basis of their presence in feces and sewage.

2. The current NSSP (National Shellfish Sanitation Program) shellfish growing area 'standard', expressed in terms of either coliforms or fecal coliforms, is based on a hypothetical relationship between the densities of coliforms and *Salmonella* spp. in a homogeneous sewage treatment plant effluent and an assumption concerning pathogen infectivity. The U. S. Public Health Service research report which established the 'standard' recognized its arbitrary nature and recommended confirmation of its validity. This has not been done.

3. Importantly, the U. S. Public Health Service report also noted that the standard was only applicable to point sources of sewage pollution. This is because assumptions regarding pathogen occurrence and ratios of pathogens to indicators only hold in sources from large populations. The presence of multiple small potential sources in nonpoint growing areas was recognized and the Public Health Service report suggested sole reliance on the numerical standard in these growing areas was not likely to provide effective public health protection. The importance of the sanitary survey and experienced judgement of an informed regulator were suggested as mechanisms to deal with such growing areas.

4. Over the past 20-25 years deficiencies with coliform and fecal coliform indicators have been noted. Departures from characteristics of an 'ideal' indicator have been described. These include prolonged persistence under favorable conditions in estuarine waters, poor persistence under other conditions, lack of source specificity, problems with recovery methodologies related to sublethal stress and culturability, the poor precision of current MPN based methods, lack of epidemiological information relating indicator numbers to risk, presence of viruses (and other pathogens) in approved waters, lack of parity between bacterial indicators and viruses coupled with observations that viruses are probably the most important etiological agents of shellfish-borne illness in recent years, etc.

5. As a consequence of Item 4., efforts have been directed toward finding and verifying 'better' indicators of fecal contamination. This has resulted in evaluations of fecal streptococci, enterococci, various anaerobic bacteria, clostridia, viruses and direct detection of pathogens. Some of these have the potential to differentiate human from nonhuman

sources. The concept of multiple indicators, including those based on chemicals, has also been suggested but inadequately evaluated.

6. Because human enteric viruses (Norwalk agent, hepatitis) appear to be the most important pathogenic agents in point source impacted growing areas, a number of investigators have proposed bacterial viruses or coliphages as candidate indicators. These viruses, which are not pathogenic to humans, offer significant advantages in terms of cost and ease of analysis. In particular, one group of coliphages, the 'male-specific' FRNA coliphages, has been identified as a promising candidate indicator. The chlorine resistance characteristics of this group are similar to hepatitis virus and Norwalk agent and may be as resistant under environmental conditions. The National Indicator Study is now funding research to examine aspects of male-specific coliphages and *Bacteroides fragilis* bacteriophage as indicators of fecal contamination in growing areas.

7. Preliminary research reports suggest male-specific bacteriophages may be useful as indicators of sewage contamination. However, its utility as an indicator in nonpoint source impacted growing areas remains equivocal and must be subjected to field verification. There is also a need to evaluate its utility as an indicator of human fecal pollution. Ideally, indicators should be sought which allow for differentiation of human from animal fecal pollution. Similarly, bacterial hosts used to enumerate these phages must be tested in natural waters.

8. The literature describing relationships between land usage and microbial pollution is rather inconclusive, especially concerning relationships between indicators and pathogens in runoff. Fecal coliforms are not specific as to source of fecal contamination and therefore cannot yet differentiate human from animal pollution in runoff, except perhaps by differences in concentrations when obviously different land usages are compared. A number of investigators have concluded the public health significance of coliform organisms in runoff remains unclear. Consequently, continued use of these microorganisms to assess water quality and public health risk remains an equivocal assumption. A priority research issue should be development of BMPs coupled with methods to effectively evaluate BMP strategies in terms of concentrations and annual outputs of human-specific fecal indicator microorganisms in runoff and receiving waters used for shellfish harvesting.

Specific research recommendations:

1. Support the National Indicator Study and other studies to identify and evaluate new indicators (focus on new indicators, rapid methods, verification via epidemiological study).

2. Support reduction of point source effluents to estuaries and improve effluent quality. Current studies indicate densities of male-specific phages are not significantly affected by chlorination, dechlorination. Their presence in sewage effluents suggests current disinfection methods are ineffective as far as viruses are concerned. Approaches to improve effluent quality by existing methods or exploration of new methods including ultrafiltration or moving effluents to ocean outfalls (as New Jersey) is needed. Evaluate use of male-specific phages released in sewage treatment plant effluents to model pollutant fields in receiving waters. Such information may be useful to address the issue of buffer zone placement.

3. Continue to support efforts to identify and validate new indicators in nonpoint source areas. Reliance on a fecal coliform numerical standard to protect public health remains equivocal. Public health would be better served by improving the scope and degreee of coverage obtained with the watershed or shoreline survey, minimizing development based on use of on site sewage disposal in areas where conditions are marginal for their installation. Bringing central sewer systems to areas where septic systems are inappropriate, and supporting in general efforts to improve the quality of surface runoff and groundwater. As long as the fecal coliform indicator continues to be used, the latter are sources of these organisms as well as nutrients that may facilitate their persistence.

4. In conjunction with Item 3., a priority research need is to perform studies to evaluate BMP strategies in terms of concentrations and annual outputs of human-specific fecal indicator microorganisms (and pathogens) in runoff and shellfish growing waters. Such studies would necessarily include evaluation of indicators in the most likely sources of fecal contamination present, i.e., animals and septic effluents, and their persistence and transport in estuaries. The effectiveness of BMP strategies to limit the impact of septic systems on sanitary water quality would be an especially important issue to evaluate.

INDICATORS OF THE SANITARY QUALITY OF SHELLFISH GROWING WATERS

Microbiological Indicators of Fecal Pollution for Use in Shellfish Growing Waters - General Comments

Historically, recognition of feces as a source of waterborne disease transmitted by the fecal-oral route occurred in Europe in the mid- to late 1800's. In the early 1900's it was generally recognized that microorganisms were more sensitive indicators of fecal contamination than chemical parameters and that direct detection of pathogens to assess water quality was fundamentally limited in usefulness because of difficulties associated with detection of multiple pathogens and their unpredictable occurrence. Use of surrogate microorganisms was considered as a reasonable means to detect the presence of fecal contamination in receiving waters. This review is generally restricted to indicators found exclusively in feces, sewage or septage that have applicability to shellfish waters. Microorganisms found in sewage or septage and which can grow saprophytically in natural waters are not included. Examples of such microorganisms are *Aeromonas hydrophila*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa.*

Although bacteria have been and are now the approved fecal indicator organism, use of fungi, and specifically the yeasts, should be briefly mentioned as they have seen limited advocacy as candidate indicators of fecal contamination. Simard (1971) discussed the use of "pink yeasts" as a possible indicator of fecal pollution, presenting various observations associating densities of yeasts with sewage pollution. On the basis of in vitro experiments which demonstrated the superior survival of *Candida albicans* compared with selected bacterial pathogens exposed to seawater, Jamieson et al. (1976) suggested this organism might be a useful indicator of fecal pollution in estuarine waters. Buck (1977) in a review of *C. albicans* as a candidate indicator of health hazards, cited deficiencies in information concerning its survival characteristics in natural receiving waters and the lack of rapid and selective enumeration methods. A "Standard Test Method" is now available based on membrane filtration and a selective medium designated mCA (ASTM 1987). Cabelli (1979) eliminated *Candida albicans* from serious consideration as a health effects indicator because of its inconsistent presence in feces and low densities. Hood (1983) failed to observe a relationship between levels of the yeast *Rhodotorula rubra* in fresh Gulf of

Mexico oysters and clams with the classification of the harvest waters, but observed that populations increased during storage suggesting possible utility as an indicator of product quality.

Issues That Affect Indicator Usefulness

The coliform group of indicator bacteria has traditionally been the basis for the microbiological growing area standard for shellfish waters since the early 1900s. Since that time researchers have identified critical deficiencies in its use as an indicator of fecal contamination or potential health risk in aquatic systems (e.g., Berg 1978; Cabelli 1978b; Dutka 1973; Shear and Gottlieb 1980). Responses to some of these criticisms are evident in a measured progression to adopt the most fecal-specific coliform organisms as approved indicators. Thus, the fecal coliform has supplanted the total coliform group, and *Escherichia coli* may replace the fecal coliform. Recent studies challenge the fundamental assumption that *E. coli* is the most efficient predictor of enteric disease risk in marine waters (Cabelli et al. 1983). Other workers have identified processes whose effects on indicator densities seriously question the validity of the coliform group as the basis for the growing water standard. Differences in resistance to disinfection and persistence characteristics of bacterial indicators compared to the most prevalent viral agents causing shellfish-associated disease continues to focus attention on the adequacy of bacterial indicators to protect public health.

As a result, interest in other indicators of fecal pollution has intensified, although information concerning the survival characteristics and recovery methodologies of alternate indicators is for the most part restricted to freshwater environments. In Section 9010 A. entitled "General Discussion" of Standard Methods (APHA 1989), the statement is made with regard to pollution in "..tidal estuaries and other bodies of saline water. " that "In the following sections, applications of specific techniques to saline water are not discussed because the methods used for fresh waters also can be used satisfactorily with saline waters." Contrary to Standard Methods (APHA 1989) there is no a priori basis to conclude that enumeration methods for freshwater will be effective in shellfish growing waters. In fact, much of the literature suggests this assumption is unwarranted.

The Indicator Concept. Use of microbiological water standards to minimize the transmission of enteric disease through consumption of raw molluscan shellfish has been a rather successful public health strategy, eliminating major outbreaks of gastroenteritis caused by salmonellae. The indicator concept, first elaborated for drinking water where contamination was assessed on the basis of an operational response using a multiple-tube enumeration procedure, was later applied to the waters of Raritan Bay in the early 1900s following incidents of typhoid associated with consumption of raw clams (Kehr et al. 1941). A target density of total coliforms was derived based on the dilution of a large point source of domestic sewage with a sufficient volume of water to yield a theoretical final ratio of indicator to bacterial pathogen. Dilution was anticipated to lower the pathogen density to a value yielding an unknown but significantly reduced potential public health risk. Use of a water-based rather than shellfish-based standard was supported by coliform data showing that bivalves (i.e., hard clams) grown in water at or below that standard indicator level would not bioconcentrate pathogens to densities exceeding a presumed minimum infective dosage. Adoption of a surrogate (indicator) for the pathogen *Salmonella typhi* was expedient for reasons that included the absence of an accurate and selective method for the recovery of this organism, and, the inability to predict its occurrence in sewage effluent. Moreover, Kehr et al. (1941) recognized the standard as an arbitrary index that does not index a predetermined level of risk, requiring verification through epidemiological investigation. "It is believed, therefore, that the most favorable method of reducing the danger of infection from the ingestion of raw hard clams is through the adoption of an arbitrary standard that would reduce to a satisfactory degree the estimated coliform content of the annual production of Raritan Bay hard clams" (Kehr et al. 1941, p. 94).

A number of basic assumptions were implicit to the indicator concept. It was assumed that the index or standard was applicable only to a diluted waste effluent, that there existed a constant density ratio of indicator to the pathogen of concern (i.e., *S. typhi*) in the effluent, and that the ratio was conserved in the environment, at least within the immediate vicinity of a discharge (Kehr et al. 1941). However, the authors recognized that if pollution sources departed from these conditions, reliance on the bacteriological standard to reflect health risk was unwarranted. Analysis of contributing sources, the interdiction of judgement, and skepticism concerning the ability of a numerical standard value to offer unequivocal health protection were aspects of their thinking.

Many authors (Bonde 1977; Cabelli 1978; Berg 1978; Wheater et al. 1980) have listed those characteristics that an ideal indicator should possess. Most have agreed that no single

indicator is "ideal" or can be universal applied. Indicators possess unique characteristics whose applicability depends on the question being asked and the specific environment involved. Thus, an indicator that is not very resistant to chlorination (a poor indicator of disinfection) would be inappropriate for sewage effluents but could be perfectly adequate in a nonpoint source polluted estuary. Very persistent indicators such as *Clostridium perfringens* spores or coprostanol may not be appropriate in a nonpoint source area but can be useful as indices of sewage plume dispersion or transport of particulate-bound material.

One paradigm of an indicator in shellfish waters would be to provide a relationship to index health risk. However, epidemiological studies to establish predictive relationships are lacking and the standards now used can reflect the presence of fecal contamination and disease-causing "potential". As noted, this usage is based on an assumed quantitative relationship between coliform bacteria and an enteric pathogen in diluted sewage from a large population. However, in nonpoint impacted estuarine and marine receiving waters, the constancy of this association may not hold because of variation inherent in small multiple sources, which are by nature intermittent, stormwater runoff, the interaction of both indicators and pathogens in the environment, and fecal pollution from nonhuman sources.

Indicators may also serve to provide information concerning source or "age" of pollution in nonpoint impacted watersheds. An indicator can be used as an investigatory tool to provide information relevant to fecal source, i.e., location within a watershed, human versus animal sources or animal type, and the age of pollution or its "freshness." Indicators inappropriate as indices of health risk may be useful for this purpose.

Persistence. Sanitary engineers and microbiologists have expended considerable effort to identify environmental factors and processes that affect densities of enteric bacteria and their recovery. Much of the early literature on coliform survival suggested that "dieoff" (or "decay") was the only functional response of coliforms exposed to marine or estuarine environments. This was primarily attributed to the "bactericidal" property of seawater (Ketchum et al. 1952). Immediate decreases in concentrations of coliform organisms discharged from a sewage treatment plant can result from physical dilution through turbulent transport and mixing, processes that presumably occur over time periods of minutes or hours. At some distance from the discharge where the effluent becomes dynamically passive, and in addition to dilution, light, temperature, bacterivory,

antagonism, inhibition, sedimentation and autecological responses become important factors affecting indicator fate.

Use of the term "dieoff" to describe changes in indicator densities over time is inappropriate. The apparent reduction of recoverable counts from estuarine waters is not only the result of "true" cell death, i.e., cells that become non-viable, but is also a function of:

1. Enumeration methodology
2. Physiological adaptation to an adverse environment
3. Complex interactions of physical, biological, and chemical processes

The net effect of these processes will be a function of their degree of interaction, relative dominance and the unique characteristics of each environment.

Initially, dilution was considered the major physical factor affecting the densities of indicator bacteria, i.e., indicator bacteria were treated as "quasi" conservative elements. We now recognize that the approved coliform indicator for shellfish growing areas does not behave as a conservative element, and can exhibit changes in density that are functions of (1) physiological responses to a variety of physical and chemical properties of the environment that may be expressed as loss of viability, change in culturability, persistence or aftergrowth, and (2) complex interactions between the indicator and components of the microbiota. In estuaries where entrainment and flushing characteristics increase contact times between indicator organisms and the environment these are important processes affecting indicator densities.

Nonfecal origin. Other deficiencies of coliform indicators include new evidence for the extraenteral origins of some species that comprise the fecal coliform group (Lopez-Torres et al. 1987), the capacity of organisms defined as fecal coliforms to persist or even grow in aquatic environments under favorable temperature and nutrient regimes (Santo Domingo et al. 1989; Hazen 1988; Knittel et al. 1977; Rhodes and Kator 1988; Verstraete and Voets 1976), the inability of the indicator to differentiate vertebrate source, the effect of environmental exposure on recoverability (Rhodes et al. 1983; Xu et al. 1982), and the recognition that allochthonous bacterial indicators are prey to microbial grazers (Anderson et al. 1983; McCambridge and McMeekin 1979, 1980; Rhodes and Kator 1988).

If these criticisms promote uncertainty about the validity of the fecal coliform indicator, they must also diminish confidence in its use as a numeric standard applicable to all shellfish growing waters. Arguably, other bacterial indicators may be found that offer advantages in terms of source specificity, determination of source age, or recovery methodology. However, considering the important influence of the environment on bacterial indicator fate and recovery, and the inherent regional and temporal variations that characterize dynamic estuarine environments, it is difficult to find credible the proposition that a standard based on a bacterial indicator can be "universally applied."

Recoverability. Another important concept concerning the fate and culturability of enteric organisms following environmental exposure was described by Xu et al. (1982). These workers noted a differential between numbers of viable cells recovered from seawater using a direct measure of viability and the viable count obtained using nonselective traditional culture methods. It was demonstrated that a significant proportion of cells starved in seawater rapidly enter a "nonrecoverable" stage, i.e., do not grow in standard media, but remain viable as shown by a direct viable count assay (Kogure et al. 1979). The phenomenon of nonculturability has been observed by other workers (Lopez-Torres et al. 1987; Munro et al. 1987; Roth et al. 1988; Martinez et al. 1989; Desmonts et al. 1990,; Garcia-Lara et al. 1991). Culturable cell counts drop significantly within 2–4 days exposure. At these times culturable counts are 20% or less of total direct viable counts. Although significant numbers of culturable cells may remain (depending on the initial cell density), these responses suggest the "true" numbers of indicator organisms could be significantly underestimated because of the low culturable densities found in approved shellfish waters. Garcia-Lara et al. (1991) employed the thymidine-labeling method of Servais et al. (1985) to demonstrate that actual mortality rates of *E. coli*, *S. typhimurium* and *S. faecium* (*E. faecium*) in natural seawater were an order of magnitude lower than mortality rates measured by culturable counts. This method involves measuring loss of radioactivity from the trichloroacetic acid-insoluble fraction (containing the DNA from intact cells) after addition of [^{3}H]thymidine-labeled cells to seawater. *E. faecium* exhibited the lowest rate of disappearance as a culturable count. Desmonts et al. (1990) combined the direct viable count (DVC) procedure of Kogure et al. (1979) with an indirect fluorescent-antibody (IFA) for *Salmonella* spp. *Salmonella* spp. were detected in all samples of raw domestic and chlorinated wastewaters by IFA-DVC at comparatively high densities, even when culturable cells were not found. Of the IFA detected cells in

chlorinated wastewater, 5–31.5% were viable by DVC. The public health significance of these studies remain to be evaluated but the relatively high counts of salmonellae observed in disinfected effluents should produce an examination of the disinfection process. Grimes and Colwell (1986) demonstrated that cells of an enterotoxigenic strain of *E. coli* became nonculturable on a BHI-synthetic seawater medium after 13 hours exposure to ocean water and remained virulent based on retention of plasmids coding for virulence genes and subsequent rabbit ileal loop assays. A detailed discussion of the viable but nonculturable phenomenon can be found in Grimes et al. (1986) and Roszak and Colwell (1987). Recognition of this physiological adaptation to starvation has done much to stimulate rethinking the fate of allochthonous bacteria in marine and estuarine environments as well as the efficacy of traditional enumeration methods. Entry of a significant proportion of an enteric indicator population during exposure to seawater into a non-recoverable stage would seriously question the validity of bacterial indicators.

However, numerous reports also demonstrate coliforms (generally *E. coli*) can persist for extended periods. Awong et al. (1990) found both wild-type and genetically engineered strains of *E. coli* survived for periods of 10-30 days, albeit with gradual reductions in culturable densities, in filtered and nonfiltered lake water. Flint (1987) reported that *E. coli* survived in sterile river water for 260 days in the absence of predators and that direct and cultural counts were similar over the first 40 days of exposure. Fiskdal et al. (1989) observed both rapid and slow declines in culturable counts of *E. coli* in filtered seawater. Extended culturability (ca. 5 months) of *E. coli* in seawater was reported by Munro et al. (1989). Munro et al. (1989) suggested that genetic differences in osmoregulatory capabilities, acting in response to the effects of test cell pretreatment and exposure conditions, are responsible for differences in survival and culturability.

We are uncertain about the causes of variation concerning entry into a nonculturable state. Some evidence suggest these may be related to properties of test strains, inocula preparation methods (e.g., Gauthier et al. 1991) or to water quality properties such as the concentration or type of organic matter. Because of the importance of this phenomenon in terms of indicator selection, its consequences on culturable enumeration methods, and recent reports that document its occurrence, a rigorous and standardized evaluation of factors that control entry of cells into the nonculturable state is desirable.

Sources of Indicators to Shellfish Growing Waters

Feces. Since the early work of Escherich (1885) who identified *Bacillus coli* (now *Escherichia coli*) as a dominant bacterium in feces, considerable effort has been expended to identify the dominant bacteria associated with the gastrointestinal tracts of humans and warm-blooded animals. Microorganisms now used as indicators of fecal contamination are necessarily (but not exclusively) inhabitants of the alimentary tracts of humans and warm-blooded animals and are not numerically dominant components of the microbiota (Savage 1977). The bacterial composition of mammalian feces, which can vary with host species, age, diet, and geographic location (Savage 1977; Feachem et al. 1983), is dominated by obligate anaerobic bacteria belonging to major taxa that include *Bacteroides, Bifidobacterium, Clostridium,* and *Eubacterium.* Commensal facultative anaerobes include the lactobacilli, *Enterobacteriacae,* and various cocci belonging to *Enterococcus* and *Streptococcus.* Concentrations of these fecal organisms range from 10^5-10^{11} organisms/g in the 150 g/day feces produced by humans in industrial societies (Feachem et al. 1983). It is estimated that up to 40% of feces is composed of microbial cells (Savage 1972). Considering that obligate anaerobes are numerically dominant in human feces (Holdeman et al. 1976; Moore and Holdeman 1974), the use of aerobic recovery methods and facultative anaerobes as fecal indicators may appear somewhat paradoxical. There is little doubt this reflects the latter's ease of recovery from aquatic environments compared to the somewhat rigorous procedures necessary for recovery of obligate anaerobes and the presumption of poor survival in oxygen-containing environments. Human and animal feces also may harbor bacteriophages that utilize bacteria as hosts. Bacteriophages that use *E. coli* as a host, e.g., somatic coliphages, F-specific coliphages and *Bacteroides fragilis* phages will be discussed in subsequent sections.

Domestic and feral animals are important potential sources of fecal contamination in urban and rural areas (Feachem et al. 1983; Geldreich 1972). The microbial composition of domestic animal feces has been examined to catalog dominant genera (Barnes 1986), to compare ratios of common indicators to those in humans (Geldreich and Kenner 1969), and to identify indicator microorganisms unique to animals (e.g., Cooper and Ramadan 1955; Geldreich and Kenner 1969; Wheater et al. 1979). Similar data for feral warm-blooded animals living adjacent to or in shellfish growing waters (e.g., harbor seals, Calambokidis et al. 1989; sea lion, Oppenheimer and Kelly 1952) are rare. Information relating the effects of animal presence on indicator and pathogen levels in adjacent estuarine receiving waters in the absence of gross pollution is needed.

Wastewaters. Anthropogenic sources of fecal organisms to shellfish growing areas include discharges of treated municipal sewage, discharges of partially treated or raw sewage that occur owing to mechanical failure, combined sewer systems, failing septic systems, and vessel discharges. Bypassing, the release of untreated sewage during periods of high rainfall, is not uncommon. Levels of bacterial indicators in effluents from a properly functioning sewage treatment plant using disinfection will be considerably reduced compared with those in the influent (Miescier and Cabelli 1982). However, disinfection is not a stoichiometric process and its effectiveness varies with waste composition, influent volume, target organism, and flow. Recent unpublished studies based on male-specific coliphages suggest chlorination of sewage effluents may not be removing the more resistant viruses (H. Kator and M. Rhodes, unpublished data; M. Sobsey, unpublished data; W. Watkins and S. Rippey; unpublished data, NETSU). Other sources include agricultural and storm water runoff, which can transport fecal wastes from humans, and domestic and feral animals to shellfish growing areas. Resuspension and transport of contaminated sediments may also be a source to growing waters. Discharges from commercial and recreational vessels into marina waters, tributaries, and growing areas have been cited as sources of fecal contamination (Faust 1982).

Nonpoint Source Runoff. Much of the literature concerning indicators in shellfish growing waters seems to have developed around the concept that fecal pollution is primarily derived from large point sources of sewage or river discharges. These rivers may integrate multiple point sources and typify conditions encountered in some highly urbanized northeastern coastal cities. Of municipal sewage facilities discharging into marine and estuarine waters, 42% are located in the northeast and these account for 60% of the total volume discharged (Shigenaka and Price 1988). Diffuse or nonpoint pollution is recognized as a major source of fecal contamination to all types of receiving waters (Geldreich et al. 1968; Gilliland and Baxter-Potter 1987; Faust 1976). Indeed, in the mid-Atlantic and Southeast regions, significant proportions of the total shellfish growing acreage are closed to direct harvesting presumably because of runoff from rural, agricultural, and wildlife sources (Leonard et al. 1989). Improperly functioning septic systems are implicated as important sources of fecal contamination. It is estimated that approximately 25-30% of households in the United States use on-site septic tank systems for treatment and disposal of wastewaters (Chen, 1988; Snowdon and Cliver, 1989; Reneau et al. 1989). The resulting per capita rate of discharge to the groundwater may

exceed 10 billion l per day of wastewater and there is reason to suspect that at least 50% of septic systems are operating marginally (Canter and Knox, 1985). Specific issues of concern are the degree of treatment afforded by such systems, which is minimal and does not inactivate viruses, and the potential migration of effluent to estuarine waters because of inadequate drainfields, poor soil characteristics, or leaching through subsurface ground water that can be affected by tidal fluctuations. A common path of septic contamination is through transport by runoff of leachate that has broken through the soil surface because of malfunctioning systems, owing to improper size, poor maintenance, inappropriate soils or water tables are common causes of this. In a study of the bacteriological quality of a recreational lake, Hendry and Toth (982) found elevated levels of indicator bacteria along the lakeshore were associated with malfunctioning or flooded home septic systems. Lower bacterial densities (below water quality target levels) were found adjacent to homes with effective systems. Chen (1988) found wastewater effluents from septic systems located on the shores of New York lakes contaminated the lakes with nutrients and fecal coliforms through infilitration by groundwater. Such contamination, which has been associated with waterborne disease in freshwaters (Vaughn and Landry, 1983; Powelson et al., 1990), may also represent a path of transport of indicator bacteria and pathogens to adjacent estuarine waters. These and other concerns are especially relevant to issues of development, where residential construction of homes using traditional septic systems may have to be curtailed and communities adopt central sanitary sewage systems. Resolution of these issues awaits the application of rigorous and imaginative experimental approaches that facilitate tracing this contamination.

The public health significance of coliform levels in growing areas contaminated by diffuse sources remains unclear. The paradigm of a diluted sewage effluent does not apply in this scenario, because sources and ratios of indicators to pathogens are expected to vary considerably and less predictably than in large sources of domestic sewage. Fecal coliform densities that exceed the growing area standard are not uncommon in Chesapeake Bay and other estuaries lacking identifiable point sources of human fecal pollution. Although direct evidence is lacking, it is believed conditions in estuaries can promote indicator survival (Erkenbrecker 1981). Contributing factors include inorganic and organic nutrient loading, high suspended solids, elevated temperatures, the presence of fine grained organic rich sediment and poor tidal flushing. Indeed, the need to identify and validate indicator systems to assess the sanitary quality of growing areas impacted by nonpoint sources may be the greatest challenge to sanitarians and shellfish microbiologists since the adoption of the coliform growing area standard. An evaluation of indicators to index health risk in non-

point source impacted areas must also consider the effects of microbial food webs and the estuarine environment on the removal and enumeration of allochthonous bacteria, the possible extraenteral origin of indicators, and contamination derived from multiple sources including wild and domestic animals. A paucity of data to evaluate these concerns has engendered criticism of the current indicator and its validity as a public health standard in these environments. However, it appears reasonable in these environments to determine if indicator data are consistent with the results of shoreline surveys.

Land Use-Effects On Sanitary Quality Of Receiving Waters.

Many studies have identified urban and rural stormwater runoff as a source of large numbers of indicator bacteria and variable levels of pathogens (e.g., Davis et al. 1977; Olivieri 1980). In general, many observers have observed the water quality of stormwaters often exceed water quality standards based on total or fecal coliform bacteria. Undetected sewage sysytem failures, wild and domestic animal feces, poor sanitation and perhaps garbage are all contributing sources. In rural areas, runoff from agricultural land has been implicated as a major source of microbial pollution to surface waters (Gilliland and Baxter-Potter 1987; Glendening 1985). Despite the importance of these sources qualitative and quantitative assessments of actual contributions and controlling mechanisms are lacking. Without such information it is difficult to evaluate the effectiveness of management strategies to reduce bacterial pollution, and the absence of real time or rapid methods to measure microbial contamination in runoff and receiving waters remains a significant limitation.

Burge and Parr (1980) reviewed the kinds of pathogenic and indicator microorganisms present in sewage and animal wastes, modes of transport to receiving waters, and aspects of indicator fate. One important conclusion was that viruses and bacteria vary greatly in their adsorptive interactions with soils. These interactions are complicated by physical and chemical processes related to specific land use, soil characteristics and water saturation, current and antecedent precipitation, temperature, age of fecal deposits, proximity to receiving waters, etc. Microbial responses (i.e., migration of microorganisms) are difficult to predict because of an imperfect understanding of these many interacting factors (Baxter-Potter and Gilliland 1988). For example, Faust (1976) found no or poor correlation between fecal coliforms discharged in runoff with nutrients, suspended sediment, or rainfall. Meiman and Kunkle (1967) claimed bacterial indicators in runoff provided a better

indication of land use than turbidity or suspended solids. Colfiorm, fecal coliform and fecal streptococci densities in runoff from nongrazed and partially-grazed lands were clearly different. Gilliland and Baxter-Potter (1987) developed a geographically-based method to predict the degree of bacterial pollution as a function of agricultural land use. Actual field data confirmed previous observations that fecal coliform densities in surface runoff consistently exceed surface water quality standards and that similar FC densities can arise from land use as different as a feed lot and a corn field (Gilliland and Baxter-Potter 1987). Faust and Goff (1977) compared annual fecal coliform discharge rates by monitoring water flow from seven watersheds that included mixtures of forest, old field, cultivated cropland, pasture, residential, and wetlands. Although fecal coliform counts were higher overall in drainage from pasture land, fecal coliform contributions expressed as fecal coliforms per ha-year were similar for all basins. Doran and Linn (1979) compared the microbiological quality of runoff from a cow-calf pastureland and a control or ungrazed land. Fecal coliform counts were 5–10 times higher in runoff from the grazed land but fecal streptococci were elevated in the control area, presumably because of feral animals. *Streptococcus bovis* was detected in streams close to the grazed area and its use to differentiate domestic from feral animal pollution was suggested. Boyer and Perry (1987) observed significant increases in fecal coliform densities in runoff from reclaimed mine land after cattle were pastured. Elevated densities were observed for several months after cattle removal and fecal coliforms survived overwintering in manure at temperatures as low as –30°C. The authors suggested that lacking information describing the comparative survival of fecal coliforms and microorganisms pathogenic to humans inder these conditions, the presence of fecal coliforms in runoff "may not be indicative of a potential health threat." Elliott and Ellis (1977) observed indicator organisms in animal wastes of various types were not always reliable determinants of pathogen presence. Burge and Parr (1980) concluded that despite the ability to differentiate runoff from lands grazed by livestock from that which supports only feral animals, the public health significance of fecal coliform indicator densities remains unknown. Gilliland and Baxter-Potter 1987) stated that attempts to judge the efficacy of mitigation activities using these indicators to measure public health risk remains equivocal. Geldreich (1981) suggested direct detection of pathogens in nonpoint pollution might be necessary to assess public health concerns. Elliot and Ellis (1977) discussed the information required to adequately assess the health hazards associated with the presence of enteric pathogens in the environment. Rather than applying the concept of zero-tolerance, public health decisions should be based on knowledge concerning the effects of environmental exposure on pathogen virulence and survival, host susceptibility, and historical incidence of disease attributed to suspect pathogens and routes

of transmission. That few managers apply this thinking is a reflection of the paucity of information required, willingness to take risks, and the conservative nature of public health regulation.

Various states have developed management strategies to reduce non-point pollution. These include notification of sewage treatment facility failure and effective warning mechanisms, BMPs to deal with animal wastes usually developed in cooperation with local soil and water conservation personnel, identification of malfunctioning on-site sewage disposal systems, correction and followup. There are very few data available relating BMPs to microbial quality of runoff or receiving waters. Doyle et al. (1975) found forest buffer strips (ca. 8 m wide) are an effective barrier against microbial stream pollution from manured fields. Levels of fecal coliforms and fecal streptococci were reduced to background levels during transport across forested buffer zones. Clausen and Meals (1989) considered the effectiveness of various BMPs on measures of water quality that included criteria for nutrients, suspended soilds, and fecal coliforms. Measured against the fecal coliform water quality criterion (200 FC/100 ml) for recreational waters), a vegetated filter strip did not reduce densities below this level in runoff. However, use of BMPs did result in overall reductions of pollutant mass output and this measure is considered as important as those based only on pollutant concentration. Clearly, new BMPs and methods to effectively evaluate BMP strategies in terms of concentrations and annual outputs of microorganisms in runoff and receiving waters should be a priority research issue.

Environmental Factors Affecting the Fate of Allochthonous Indicators of Fecal Contamination

Effects of various environmental parameters on indicator survival have been examined by many investigators but it is often difficult to unequivocally assess their "true" significance. Concerns arise with studies based on the use of in vitro experiments, laboratory-adapted strains, artificial or filtered menstrua, and exposure devices incapable of preventing external contamination or containment of test strains (Roper and Marshall 1979; Anderson et al. 1983). Given the probability that the microbiota can be a major factor affecting bacterial numbers, it seems unwise to generalize based on experimental designs that exclude this component. This is not to discredit the value of in vitro studies, which are useful to establish basic principles, but ecological hypotheses should be tested under conditions that duplicate open or natural systems as closely as possible. Another concern focuses on

methods used for preparation of test cells. A majority of survival studies involved procedures for preparation of test cells known to result in sublethal stress or be physiologically debilitating. Culture age, growth conditions, and laboratory manipulations are important contributing factors contributing to sublethal injury. Use of cells pregrown in rich media and harvested during the exponential phase of growth, cold-shocking, and harvesting by repeated centrifugation in unfavorable solutions are known to compromise physiological indices, increase sublethal stress, reduce adenylate levels, reduce enzyme activities, produce changes in membrane integrity leading to leakage of cell constituents, and render cells sensitive to free radicals or heavy metals (Postgate 1967; Strange 1976; Anderson et al. 1979; Granai and Sjogren 1981; Rhodes et al. 1983). Failure to recognize these factors as sources of experimental bias has led to incorrect inferences of causality. Similar reservations apply to using laboratory adapted strains because these cells may exhibit survival characteristics that are "atypical" compared with "fresh" fecal isolates (Anderson et al. 1979).

In view of the importance accorded test cell preparation, a relevant hypothesis that should be tested concerns the effect of indicator origin (exposure prehistory) on its fate. Are there differences in the survival of indicator cells prepared under laboratory conditions and cells derived from "natural" sources such as sewage treatment plant (STP) effluent, septic tank effluent, agricultural or stormwater runoff? The STP or septic system provides a nutrient rich environment, at times maintained at a higher temperature than the receiving waters, and exposes cells to varying degrees of toxic materials or disinfectant-mediated injury. Could it be that indicator bacteria from diffuse natural sources are physiologically adapted to adverse nutrient concentrations, intermittent lack of water, unfavorable temperatures, etc? Such adaptation could indicator persistence and recoverability compared with laboratory-grown cells.

Finally, it is difficult to see how investigations that treat the effects of environmental factors as if they were independent operators can lead to developing basic principles of indicator cell survival. Treatments that isolate single variables will only provide information concerning the potential role of that variable. In the environment survival will be affected by the interaction of that factor with perhaps more dominant processes. Laboratory studies must be augmented by admittedly more complex and variable in situ exposure studies that integrate physicochemical and biological factors. Conversely, in situ experiments must be carefully interpreted because of the potential for undetected processes that may affect test cell densities. Examples include breaching of exposure devices by autochthonous

organisms owing to design or structural damage (Roper and Marshall 1979; Anderson et al. 1983) and physical penetration through the membrane "pores" by bacterivorous nannoflagellates (Cynar et al. 1985) or other procaryotes (Li and Dickie 1985). Chambers may exhibit highly variable "bottle" effects because of nutrients contributed by fouling communities on chamber surfaces, blooms caused by exclusion of autochthonous organisms or predators, and attenuation of incident light. Finally, estuaries are unique dynamic systems, characterized by temporal and spatial heterogeneity. Generalizations based on experiments performed in Puget Sound may not be applicable to Chesapeake Bay or Gulf of Mexico growing waters.

The following sections summarize important aspects of the effects of physical, chemical and biological factors on indicator survival, primarily under estuarine and marine conditions.

Physical and Chemical Factors

Temperature. At first glance the literature dealing with the role of temperature on the in situ survival of *E. coli* in saline waters appears equivocal. Investigators have reported both positive (Anderson et al. 1983; Rhodes and Kator 1988) and negative (Faust et al. 1975; Vasconcelos and Swartz 1976; Lessard and Sieburth 1983) correlations of temperature and survival. This confusion can be resolved if it is understood that temperature has both indirect and direct effects on indicator fate. It can have a direct effect on bacterial activity, so that under appropriate conditions multiplication may occur at warmer seasonal temperatures. *E. coli* cells prepared under conditions to minimize stress initially exhibit multiplication (large negative values of the mortality rate coefficient, k) in membrane filtered water (0.2 μm) at elevated temperatures in Chesapeake Bay (Rhodes and Kator 1988). Maximum negative values of k correspond to increases in viable counts of approximately 1.0 to 1.5 log units. Although it may be thought that low environmental temperatures (<10°C) would reduce coliform metabolic activity based on Q values, thereby favoring persistence, low environmental temperatures do not appear to have this effect as claimed by some authors. The indirect effects of low environmental temperature will be addressed in the context of sublethal stress but exposure of bacterial cells to low temperatures is known to compromise cell envelope integrity and physiological indices (Strange 1976). Indeed, positive values of k were obtained at temperatures below 10°C in filtered estuarine water (Rhodes and Kator 1988).

An indirect and highly significant relationship between temperature and indicator recovery and enumeration is the effect of temperature on the development of sublethal stress. Sublethal stress reflects the inability of a microorganism to be cultured in a medium because of prior injury, impairment or damage that resulted from exposure to unfavorable environmental conditions. Sublethally stressed cells are particularly sensitive to recovery methodologies that use selective temperatures, lack resuscitative protocols or use inhibitory substances to enhance media specificity and selectivity (Hackney et al. 1979). We have quantified the development of sublethal stress in *E. coli* as a function of both temperature and salinity (Anderson et al. 1979; Rhodes et al. 1983). Sublethal stress and mortality are inversely related to temperature. At temperatures below 10°C transiently acute or progressive development of sublethal stress is detectable using a variety of techniques. These include differential counts on selective vs. non-selective recovery media and an assay technique (Anderson et al. 1979) based on the observation that sublethally stressed cells require a longer period of time to produce an electrochemically-induced potential difference compared with non-stressed cells [electrochemical detection time (EDT)]. In practical terms, sublethally stressed cells may not be detected using enumeration methods that incorporate selective procedures. The effect of exposure to estuarine water on enumeration efficiency using experimental and approved recovery methods (Rhodes et al. 1983) causes progressive stress over time even with favorable environmental temperatures. Selective methods such as the direct M-FC procedure, an approved method (APHA 1989), exhibit poor enumeration efficiency. The rosolic acid in this medium significantly reduces the recoverability of chlorine-injured fecal coliforms from sewage (Presswood and Strong 1978). Although resuscitation procedures in non-selective media have been employed to diminish the impact of selective enumeration, such measures may not be completely effective.

Sublethal stress is an important phenomenon that must be considered if conventional selective enumeration procedures are used for the recovery of indicator cells. Results from survival studies with *E. coli* where viable counts were not "corrected" for the effects of sublethal stress tend to overestimate mortality (underestimate cell densities), with the error being most significant at temperatures below 10°C. As a result, the responses of cells to physical parameters or treatments where sublethal injury was unrecognized must be interpreted with caution because the observed mortality may have been incorrectly attributed to another variable and not the enumeration process. These concerns reinforce the notion

that sublethal stress is an important factor affecting indicator choice when viable recovery methods are used and observed cell densities are to have regulatory significance.

Temperature-induced sublethal stress also rendered cells sensitive to other stressors. Mackey and Derrick (1986) presented evidence that cold-shock sensitized *E. coli* to very low concentrations of hydrogen peroxide and as a consequence reduced recovery of viable cells on an organic-rich medium. Postgate (1967) cautioned against chilling of samples and cultures as a practice that promoted the death of stressed cells. Jackson (1974) observed loss of viability of *Staphylococcus aureus* and increased sensitivity to a selective medium at 5°C and noted *E. coli* became more sensitive to violet-red-bile agar exposed at this temperature. The implications of these observations apparently remain unheeded because Standard Methods (APHA 1989) suggests icing of microbiological samples if they cannot be processed within 1 hour. A systematic examination of sample storage parameters to optimize recovery under a variety of seasonal temperature regimes should be part of the indicator evaluation process.

Indirect effects of temperature on coliform fate arise from the influence of seasonal temperature on the densities, composition and activities of the indigenous microbiota (Verstraete and Voets 1976; Anderson et al. 1983; Rhodes and Kator 1988). The role of the microbiota on indicator persistence and survival has been until recently a topic of considerable speculation. Thus, although the activities of antagonistic substances, parasitic and lytic microorganisms, and protozoans were recognized as potentially important factors controlling indicator abundance (Mitchell and Yankofsky 1969; Mitchell 1972; Mitchell and Chamberlin 1975; Drake and Tsuchiya 1976), it is only recently that microbial ecologists have identified bacterivory as important carbon and energy pathways (Wright and Coffin 1984). The importance of bacteria in coastal waters as food for heterotrophic microflagellates has been demonstrated (Fenchel 1982; Anderson and Fenchel 1985). Using diffusion chambers, Awong et al. (1990) observed that densities of genetically engineered strains of *E. coli* were significantly reduced in nonsterile lake water compared with cells in filter-sterilized water. Gonzalez et al. (1990) observed bacterivory of *E. coli* and *E. faecalis* by natural assemblages of estuarine flagellates and ciliates incubated in Whirl-pak bags. Both predators groups exhibited grazing preference for large bacterial cells but ciliates ingested *E. faecalis* at a higher rate than *E. coli*. Studies with diffusion chambers, perhaps somewhat artifactual in terms of community development and enhanced encounters between predator and prey, have revealed the potential influence of the microbiota on *E. coli* survival in estuarine water (Rhodes and Kator 1988). Generally,

times of maximum decline in nonfiltered water coincided with maximum densities of heterotrophic flagellates or other predators (Rhodes and Kator 1988). Although predation and other microbially-mediated removal processes, and the "intrinsic" growth responses of enteric bacteria were direct functions of temperature, the net combined effect of increased temperature was indicator removal. It is evident that failure to recognize both direct and indirect effects of temperature has lead to the erroneous conclusion that elevated temperature, per se, does not favor coliform survival.

Salinity. Efforts to determine the effect of salinity on indicator survival constitutes a small and somewhat inconclusive literature (Carlucci and Pramer 1960; Faust et al. 1975; Orlob 1956; Vasconcelos and Swartz 1976). Results reported, which are generally for *E. coli*, range from no effect of salinity on *E. coli* viability to reduced survival with increasing salinity. This literature remains problematic for many of the same reasons noted for temperature studies. The combination of all-or-none viable counting methods with factors known to be stressors, e.g., selective recovery methods, artificial seawater with possible trace toxicants, harsh inocula preparation methods, contributed to the apparent disappearance of test cells. These problems were avoided with techniques that measured graded responses and used cell preparation techniques that minimized injury (Anderson et al. 1979). Graded responses included EDT in selective and non-selective media, β-galactosidase specific activity, and growth rate. Increased salinity was accompanied by decreased recovery of viable cells, an increase in sublethal stress expressed both in terms of larger EDT values and as the difference in cell recovery on selective versus non-selective media, and as a marked reduction in β-galactosidase specific activity (Anderson et al. 1979). Cells exposed to 30 psu seawater for 2-9 days exhibited an apparent mortality about 9 times greater when recovered in EC medium than the same cells recovered in TSB. The idea that exposure of *E. coli* to saline water yields cells with altered physiological properties, thereby requiring modified enumeration methods, has been suggested. Dawe and Penrose (1978) observed that salinity-induced debility in coliforms is reduced by incorporation of seawater into the recovery medium. Gauthier et al. (1987) increased recovery of *E. coli* cells adapted to seawater by including sodium chloride in an enumeration medium, although the degree of response was strain and time dependent. Munro et al. (1987) observed that *E. coli* starved in seawater manifested a variety of physiological and structural responses including loss of β-galactosidase activity, increased activity of other enzymes, and altered sensitivities to antibiotics, phages and heavy metals. Increased enzyme activity toward 4-methylumbelliferyl heptanoate observed during

starvation of *E. coli* in seawater was presumably a response to low nutrient conditions (Fiksdal et al. 1989). *E. coli* cell envelope composition and functional characteristics are different in cells grown in an estuarine water-based medium compared with those grown in a standard medium (Chai 1983). The effect of high osmolarity in *E. coli* is to repress synthesis of OmpF porin protein, a protective mechanism to reduce cell permeability to naturally occurring detergents, i.e., bile salts (Nikaido and Vaara 1987). This adaptative process retains membrane permeability, albeit reduced, to nutrients with molecular weights of 100-200. An indirect consequence of reduced membrane permeability under hyperosmolar conditions could be to alter significantly or reduce the uptake of molecules used in direct cell viability assays such as the tetrazolium salts. Gauthier et al. (1991) discussed the significance of cell preparation methods on *E. coli* survival in seawater. Procedures causing loss of intracellular K^+ and glutamate affect the ability of cells to regulate osmotic pressure and reduce their resistance and survival in seawater. Restoration of intracellular K^+ and glutamate restores regulation of cellular osmotic pressure and enhances survival in seawater. In summary, the studies mentioned support an emerging hypothesis that survival of *E. coli* in seawater is an active process involving physiological adaptation to an adverse environment. Recovery of adapted cells may be optimized using a resuscitative environment that allows cells to readapt to conditions of lower osmolarity.

Strange (1976) in his monograph on stress noted that osmotic shock causes loss of cell viability, reduces active transport of solutes, and releases a variety of metabolites and enzymes. Roth et al. (1988) demonstrated that a large proportion of *E. coli* cells exposed to hyperosmotic conditions are not recoverable on a non-selective medium. However, if the cells are osmotically upshocked in the presence of betaine (N, N, N-trimethylglycine, a naturally occurring and ubiquitous nitrogen-containing compound produced by microorganisms, animals and plants), the cells remain culturable. Betaine accelerates uptake of ATP and reduces intracellular ATP that accumulates in osmotically upshocked cells and facilitates protein synthesis. Conjugative transfer of a plasmid between donor and recipient *E. coli* cells was also enhanced by glycine betaine in autoclaved sediments (Breittmayer and Gauthier 1990). Roth et al. (1988) developed a resuscitation method using a medium incorporating betaine, chloramphenicol (to prevent changes in cell density during resuscitation), ammonium and glucose as nitrogen and carbon sources. At this writing we are unaware if this method has been evaluated with estuarine samples.

Adsorption and Sedimentation. The effect of estuarine particulates on the persistence of autochthonous bacteria continues to be an interesting area of research. Rubentschik et al. (1936) suggested that adsorption of *E. coli* to particulates with subsequent deposition in sediments is an important aspect of self-purification in salt lakes. Weiss (1951) investigated *E. coli* adsorption on river and estuarine silts and concluded that adsorption enhances bacterial sedimentation rate and is a function of particle type and size. Although flocculation of silts increase in seawater, the adsorptive capacity of silts toward bacteria decreases with increased salinity and the bacteria desorb. Milne et al. (1986) concluded that removal of fecal coliforms from estuarine water by deposition was directly related to the concentration of naturally-occurring suspended solids. A similar relationship was not observed in seawater and attributed to the differences in the depositional behavior of suspended solids in the two systems. Roper and Marshall (1974, 1979) observed that *E. coli* adsorb to sediments at high electrolyte concentrations and desorb below a critical concentration. They hypothesized that in low electrolyte (freshwater) systems electrostatic forces allow bacteria to exist as stable colloidal dispersions. As salinity is increased, flocculation and sedimentation of bacteria and particles will occur. This straightforward model can be complicated by organics present on the microorganism's "surface." Thus, adsorbed bacteria can adhere very strongly to sediment particles owing to production of organic exopolymers (Marshall, 1985). Viruses also adsorb to estuarine sediments. Labelle and Gerba (1979) observed >99% adsorption of various enteric viruses to sediment at salinities of 1–35 psu. Alterations in salinity and pH produce small but variable effects on adsorption and desorption of most virus types examined. Because enteric viruses did not readily desorb they concluded that viral transport would be dependent on particle resuspension and transport.

Based on the above observations it is not surprising that densities of fecal indicator bacteria (Erkenbrecker, 1981; Shiaris et al., 1987) and enteric viruses (LaBelle et al., 1980, Rao et al., 1984) are elevated in estuarine sediments compared to overlying waters. Sediments from an urban shellfish growing area in the Chesapeake Bay contained fecal coliforms at densities two orders of magnitude larger than in the water column (Erkenbrecker 1981). Variable but generally smaller values of the ratio of fecal coliform densities in sediment to water were observed in a small subestuary subject to nonpoint pollution (Kator and Rhodes 1989). LaBelle et al. (1980) found no correlation between densities of bacterial indicators and virus in estuarine waters. There was a positive correlation between the numbers of viruses and fecal coliforms in sediments.

In vitro and in situ experiments (Gerba and McLeod 1976; Roper and Marshall 1974, 1979; Peresz-Rosas and Hazen 1988) have shown the positive effect of marine or estuarine sediments on *E. coli* persistence. Similarly, adsorption to estuarine sediments enhances survival of enteroviruses (Smith et al. 1978; Toranzo et al. 1982) and bacteriophage T7 (Bitton and Marshall 1974). Protective effects are attributed primarily to accumulation of nutrients on particle surfaces and reduced predation (Roper and Marshall 1978) and antibiosis. As previously mentioned, Roth et al. (1988) reported the organic compound betaine minimizes osmotic stress and restores the culturability of *E. coli* exposed to seawater. Le Rudulier and Bouillard (1983) have shown that betaine concentrations as low as 1 mM eliminate osmotic stress in *K. pneumoniae*, *S. typhimurium* and *E. coli* over a range of high NaCl concentrations (0.65-1.0 M). There was no evidence that betaine is utilized as a growth substrate in *K. pneumoniae*. Betaine is a ubiquitous and relatively abundant compound in benthic organisms and is an important substrate in fermentation pathways coupled to methanogenesis in marine sediments (King 1984; King 1988; Heijthuijsen and Hansen 1989). The apparent enrichment and persistence of *E. coli* (or other enteric bacteria) in sediments may be augmented by the osmotolerance afforded by betaine or other osmolytes (Munro et al. 1989; Gauthier and Le Rudulier, 1990; Ghoul et al. 1990). The effects of anaerobic sediments, which are sources of inorganic nutrients and small molecular weight fermentation products, on indicator persistence is obviously a research issue requiring further study.

The accumulation and enhanced survival of sewage microorganisms in sediments have been employed as a justification for questioning the validity of using a water quality criterion as the basis for classification of shellfish growing waters (Shiaris et al. 1987). Sediments provide a more integrated recent history of fecal pollution than overlying waters that reflect transient pollution events. Relationships between indicators in sediment and river water were examined in a study by Matson et al. (1978). Sediment deposition, resuspension, and transport were identified as processes that significantly affect densities of indicators (and enteric pathogens) in overlying waters (Matson et al. 1978; LaBelle et al. 1980; Erkenbrecker 1981). Thus, the absence of microorganisms from the water column may not reflect a diminished health hazard. Metcalf et al. (1973) noted that fecal coliform concentrations and the likelihood of isolating salmonellae in the water column were functions of tidal stage, suggesting an association between tidal currents and the resuspension and transport of particulate material. Particle-associated pathogen transport is emphasized by greater viral accumulation in shellfish exposed to resuspended sediment as

compared to those placed in a system containing undisturbed sediment (Landry et al. 1983).

Light. Direct lethal effects of light on enteric bacteria in seawater have been demonstrated under in situ (Gameson and Saxon 1967; Gameson and Gould 1975; Bellair et al. 1977; Fujioka et al. 1981) and in vitro experimental treatments (Gameson and Gould 1975; Fujioka et al. 1981; Kapuscinski and Mitchell 1981; McCambridge and McMeekin 1981; Fujioka and Siwak 1987; Cornax et al. 1990. Tartera et al. (1988) compared the sensitivities of bacterial and viral indicators to UV light under in vitro conditions. Coliphage f2 and a *Bacteroides fragilis* bacteriophage are more resistant than *E. coli* and *E. faecalis*. Male-specific coliphages are significantly more resistant to UV radiation than indicator bacteria (Havelaar 1986; 1987). The overall importance of these studies to the mortality of indicator organisms in shellfish growing waters remains somewhat equivocal. This is because estuaries where shellfish are produced, for example in the eastern United States and Gulf of Mexico, are highly turbid and can be dominated by complex microbial food webs. The importance of these factors resides in the attenuating capacity of suspended and dissolved material toward lethal wavelengths of light and the light-stimulating effect on components of the microbiota that can lead to enhanced bacterial mortality. Attenuation of UV-B (280-320 nm) light may occur in the uppermost layer of the water column although the degree of attenuation in coastal waters is influenced by local properties that affect light absorption such as dissolved organic matter, chlorophyll concentration and particulate load (Calkins 1982). Interactive effects of light and the autochthonous microbiota have been demonstrated in fresh and estuarine waters under in vitro conditions (McCambridge and McMeekin 1981; Barcina et al. 1989). A series of in situ experiments were performed in the Chesapeake Bay using diffusion chambers specially modified to maximize light penetration and employing light and dark, filtered and nonfiltered treatments (Rhodes and Kator 1990). Compared with cells suspended at 1.0 cm below the water surface, mortality from sunlight was essentially insignificant at 25.0 cm except during periods (fall, winter) of minimal light attenuation. Cells at 1.0 cm also exhibited significant sublethal stress. During the warm seasons significantly greater mortality occurred in the presence of light and the microbiota than with either alone. Enhanced mortality of the combined treatment can be attributed to stimulation of predation, by sunlight, light-dependent release of antagonistic substances, or formation of photochemically induced toxicants. Therefore, in turbid shellfish growing waters sunlight-

induced injury and mortality result from direct and indirect effects whose relative influence will be a function of local conditions.

Organic Compounds and Nutrients. In a review of factors affecting the survival of enteric microorganisms in marine and estuarine environments, Mitchell and Chamberlin (1975, p. 241) concluded a section on "Nutrient deficiencies" as follows: "These results coupled with laboratory findings tend to suggest a significant role for nutrients in determining survival of enteric bacteria in seawater". Until recently the literature did not refute this conclusion, but did little to amplify it or provide data supporting this hypothesis. Fortunately, this area has been the focus of a number of research groups such as Lopez-Torres, et al. (1987), who describe a positive statistical association between nutrient concentrations and persistence of *E. coli*. *E. coli* starved in seawater manifest a variety of physiological and structural responses including changes in enzyme activity, and altered sensitivities to antibiotics, phages and heavy metals (Munro et al. 1987). Fiksdal et al. (1989) concluded increased hydrolase activity toward 4-methylumbelliferyl heptanoate by *E. coli* starved in seawater is an adaptation to nutrient limitation. Studies are still needed to evaluate the direct effects of inorganic nutrients and organic carbon on enteric survival, especially in nutrient-rich shellfish growing areas that are not well flushed, where high productivity associated with detrital food webs, allochthonous inputs, and organically enriched fine sediments may provide nutrient conditions favoring indicator persistence.

Toxic Compounds. Although the lethality of disinfectants toward indicator bacteria is well described, the effects of toxicants present in shellfish growing waters and sediment on these microorganisms is a rather impoverished area of research. This is somewhat surprising considering that many estuarine areas are polluted by high levels of toxic heavy metals, xenobiotics and other materials. Runoff from agricultural and urban areas contains a variety of pesticides of varied toxicity and persistence characteristics. Elevated concentrations of zinc have been found in coastal sediments impacted by sewage discharges (Bruland et al. 1974). Effluents from kraft pulp mill plants may elicit a broad range of toxic effects (Sodergren 1989). Oil shale process waters are lethal to coliforms and *S. faecalis* under in vitro exposure conditions (Adams and Farrier 1982). Jones and Cobet (1975) noted the toxicity of naturally occurring heavy metals in Caribbean seawater to enteric bacteria. Widespread use of organotin compounds as antifouling paints has been recognized as contributing to high tributyltin concentrations in estuarine waters and

sediments frequently exposed to vessels. Pettibone and Cooney (1986) concluded that organotin compounds are not acutely toxic to *E. coli* and *E. faecalis* isolates at naturally-occurring concentrations but act as stressors. Chai (1983) noted that *E. coli* grown in a medium containing estuarine water manifested changes (adaptations) in cell envelope composition, changes that afforded decreased sensitivity to bacteriophage infection and colicins but also rendered cells more sensitive to heavy metals and detergents. In a series of experiments to evaluate the effects of 10 mM Zn^{2+} on bacteria and selected coliphages, the toxicity of zinc to *E. coli* was increased in the presence of 0.1 M or higher sodium chloride (Babich and Stotzky 1978). In vitro exposure of *E. coli* to estuarine water containing various toxic chemicals alters cell envelope protein composition, detectability of plasmids, and affects carbohydrate and amino acid metabolism (Palmer et al. 1984). Creosote contamination of estuarine sediments is detrimental to microbial communities as reflected in reduced secondary production and biomass (Koepfler and Kator 1986). Although definitive information is lacking, sediments may be sources of chemical and biological stressors toward indicator bacteria although it is likely the degree of effect will be site specific.

Biological Factors

Biological factors that affect indicator fate have been discussed or mentioned in other sections. A complete survey of this literature is beyond the scope of this review. Biological interactions between indicators (bacteria or viruses) and components of the microbiota are complex and definitive in situ rate measurements describing interactive processes are lacking. Processes effecting removal through predation, parasitism or lytic activity (e.g. Berk et al. 1976; Roper and Marshall 1977, 1978; Enzinger and Cooper 1976; Anderson et al. 1983; Rhodes and Kator 1988) or antibiosis (Sieburth and Pratt 1962; Moebus 1972; Aubert et al. 1975; Toranzo et al. 1982; Girones et al. 1989), and competition for nutrients (Jannasch 1966) have been described. A need exists to determine the relative importance of biological interactions compared with other processes that affect indicator survival or removal. Because biological processes in estuaries are highly variable in temporal and spatial dimensions, studies to assess these effects must be performed with sufficient replication and seasonal coverage to ensure the collection of representative data. Technical impediments to in situ experimentation that require resolution are uncontrolled contamination of exposure chambers by autochthonous microorganisms, inadequate sample volumes, and the need to unequivocally differentiate test cells from the autochthonous cells.

Garcia-Lara et al. (1991) proposed use of [^{3}H]thymidine-labeled cells to evaluate the overall contribution of grazing and lytic processes on indicator mortality. Analytical approaches combining rapid direct viable counting methods with those permitting unequivocal identification of test organisms (e.g., fluorescent antibody, gene probes, etc., Roszak and Colwell 1987) may also provide solutions.

INDICATORS, THEIR DETECTION AND ENUMERATION

Traditional Indicators for Water

Coliforms. Historically coliforms have evolved to become approved indicators of sanitary water quality for drinking, surface and estuarine receiving waters. During this period, methods for their recovery and enumeration have been under continual scrutiny, yielding refinements in selectivity, specificity, and efficiency characteristics. Over the last several decades the effects of environmental exposure on coliform recovery have been recognized and repair procedures evaluated. The 17th edition of Standard Methods (1989) recognizes injury as a factor affecting recovery of coliform indicator bacteria, suggesting a variety of steps to enhance recovery.

Although the total coliform group can be found in most current compilations of approved microbiological methods, the overwhelming thrust of literature dealing with indicators, relationships between indicators and pathogens and the epidemiological verification of indicators is rather clear in authenticating the extrafecal origins and growth potentials of members of this group, the superior specificity of the fecal coliform as an indicator of feces, and its lack of correlation with pathogens and health effects. Based on these facts one must conclude that although the group remains an accepted indicator and standard for drinking water, its continued application to shellfish growing waters is not justified.

Although as previously noted the validity of total coliforms as an indicator of fecal contamination in natural shellfish waters is equivocal, use of the total coliform group to classify shellfish growing waters is still recognized by the National Shellfish Sanitation Program (1990) and therefore methods for its recovery are included in this review. Approved methods (APHA, 1989) for enumeration of the coliform group of bacteria from shellfish waters include multiple-tube fermentation and membrane filtration techniques.

Each method has its unique characteristics and advantages. The multiple-tube fermentation method (MPN) is comparatively simple to perform but mechanically repetitious, and using 3 or 5 tubes per dilution (most commonly used) provides a relatively imprecise estimate of the density of coliforms in a given sample. However, the MPN method does allow for processing samples that contain relatively high amounts of suspended particulates, chlorine, or other toxic chemicals compared with membrane filtration.

The total coliform group consists of all aerobic and facultative anaerobic, gram negative, non-spore-forming rod-shaped bacteria that ferment lactose with gas and acid formation within 48 h at 35°C (APHA, 1989). Approved methods call for incubation first in lauryl sulfate tryptose broth, although lactose broth may be used, followed by transfer of tubes producing gas to brilliant green lactose bile broth, etc. Approved MPN-based methods do suffer from interferences caused by the presence of microorganisms that inhibit gas production or are antagonistic to coliforms (Evans et al. 1981). Coliform masking can be intensified with sublethally stressed cells and was demonstrated with seawater samples (Olson 1978). Some coliforms may be anaerogenic and therefore tubes with growth should be confirmed through additional and time consuming testing (Olson 1978; Evans et al. 1981). Another source of interference are organisms such as *Aeromonas* spp. which produce gas in standard media or can overgrow coliforms. These effects can lead to underestimation of coliform densities or failure to detect their presence.

Total coliforms may also be enumerated using a membrane filter approach (Geldreich 1981). The latest edition of Standard Methods (APHA, 1989) offers an approved total coliform membrane method (using either M-Endo or LES Endo agars) and also presents a method for recovery of injured total coliform bacteria as well as an abbreviated discussion of stress. Avila et al. (1989) recommended M-Endo as the most suitable medium for recovery of coliforms from seawater by membrane filtration. The membrane method offers better reproducibility, sensitivity and precision than the MPN approach (Clark et al. 1951). However, the obvious advantage of sample concentration leads to problems associated with high turbidity, injury owing to adsorption of chlorine or other toxics by the filter, and concentration of noncoliform bacteria. Interference caused by the physical presence of background microorganisms or even the production of coliform-specific bacteriocin-like substances has been considered (Means and Olson 1981; Burlingham et al. 1984). The volume filtered in saline waters may be restricted because of high particulate concentrations which interfere with colony formation and counting. Geldreich (1981) and Hartman et al. (1966) consider basic problems associated with membrane filtration-based methods.

Fecal Coliform. The fecal coliform group, the most frequently used indicator of fecal contamination to classify shellfish growing waters, replaced the total coliform group as a more specific indicator of fecal pollution in natural waters. However, it also possesses characteristics that have engendered criticism of the total coliform group. The fecal coliform group is operationally defined (APHA 1985) and includes genera that are not restricted to fecal habitats and may grow in saline receiving waters. The standard procedure for enumerating fecal coliforms does not differentially resolve component genera. By extension, if the numeric value of the total coliform standard was not based on a quantitative assessment of health risk, then the same must be said of the fecal coliform standard, the latter derived by correlative analysis of paired total and fecal coliform data from growing areas (Hunt and Springer 1974). The discrete value chosen also reflects a methodological bias dictated by the constraints of the MPN distribution table. Compared with the enterococci, the fecal coliform indicator (and *E. coli*) was considered an inferior indicator of sewage pollution because of large reductions in its density that occur during sewage treatment, its greater sensitivity to chlorination, and its higher rate of dieoff (Miescier and Cabelli 1982). *E. coli* has been shown capable of persistence for periods of extended duration, or even aftergrowth in estuarine waters. It is noteworthy that shellfish harvested from approved growing areas in the Gulf of Mexico during periods of seasonally warm temperatures may be rejected because fecal coliforms levels in the meats exceeded the market guideline owing to multiplication of fecal coliforms after harvesting (FDA 1984). Recent reports note how its entry into a nonculturable but viable physiological state, its physiological adaptation to seawater, and the effects of sublethal stress can lead to significant underestimation of its numbers in seawater. Sublethal stress can have a significant effect on bacterial recovery at environmental temperatures below 10°C, in waters of high salinity, in the presence of toxic chemicals, or due to light-induced damaged.

An alternative to the APHA MPN method (APHA 1985) is the approved 24 h modified A-1 MPN method (Hunt and Springer 1978; APHA 1985) method. For better precision a membrane filter direct counting method based on the use of mFC agar is approved for use (APHA 1989) but is subject to the same limitations discussed for the total coliform membrane filter method. Use of rosolic acid may be inhibitory to injured coliforms and may be omitted (Geldreich 1981). Pagel et al. (1982) found recovery of fecal coliforms on mTEC agar, a medium designed for selective recovery of *E. coli*, is superior in overall performance characteristics to mFC. A alternate filter-based approach is the hydrophobic

grid membrane filter (e. g., Sharpe 1981) coupled with an appropriate selective media such as described by Entis and Boleszczuk (1990). These filters accommodate an increased range of counts, i. e., 3 orders of magnitude compared with ordinary membrane filters, and provide considerably better precision than the MPN method. Waxed grid lines used to divide the membrane into hundreds of independent compartments or cells, confine colony growth, prevent spreading and inhibition by background bacteria.

Unapproved or Emergent Methods for Enumeration of Coliforms and Fecal Coliforms. Methods discussed in this section have been developed to enumerate the target microorganisms within a significantly shorter time interval than required for approved APHA MPN methods (APHA 1989, APHA 1985). The methods can be arbitrarily classified into approaches that are modifications of the existing enumeration procedures but use different media or procedures, those that combine different media with a variety of techniques to detect activity or densities of viable cells, and those that are based on direct detection of cellular nucleic acids. Additional variations may be obtained by combining these three categories. A recent review by Boardman et al. (1989) discusses new methods for detection of coliform bacteria and other indicators found in marine waters.

Kamplemacher et al. (1976) compared the existing APHA MPN enrichment method for detection of total coliforms with three enrichment media used in Europe and also compared direct recovery of *E. coli* from these same media. One medium, formate glutamate, was found to be result in higher recoveries and significantly fewer false positives after 48 h than LST broth.

A variety of rapid enumeration methods for coliforms and fecal coliforms are referred to in Standard Methods as Special Techniques (APHA 1989). A table of methods with appropriate references is provided (APHA 1989) and include radiometric, enzyme assay, electrochemical, impedance, gas chromatographic, colorimetric and potentiometric assays. Many of these techniques are generally considered inappropriate for routine enumeration of fecal coliforms in water samples. Only methods based on the colorimetric enzyme assays and ^{14}C-labeled substrates are recommended by the APHA (1989). All these methods are calibrated against standard curves obtained by viable count methods. This can lead to problems of interpretation and large errors because the responses obtained with environmentally stressed and substrate non-responsive cells complicates interpretation of standard curves.

Berg and Fiksdal (1988) evaluated three fluorogenic substrates, 4-methylumbelliferyl-β-D-galactoside, 4-methylumbelliferyl-heptanoate, and 4-methylumbelliferyl-β-D-glucuronide for a rapid method to detect total and fecal coliforms based on enzyme activity. Samples of river water, potable water, or sewage effluents were filtered through 0.45 μm membrane filters, the filters incubated in a nutrient broth containing a fluorogenic substrate, lactose, sodium lauryl sulfate, and buffer at temperatures selective for each coliform group. Initial rates of substrate hydrolysis, measured over time by fluorescence, were related to initial coliform densities. Linearity of fluorescence over the first hour of incubation was very good. Of the substrates examined 4-methylumbelliferyl-β-D-galactoside gave the best response time, providing a positive result in 15 minutes. Incorporating this substrate in M 7h FC agar (APHA 1989) provided a direct method for enumeration of fecal coliforms within 6 hours.

Escherichia coli. This organism is considered the dominant fecal coliform in human and animal feces and is generally considered an indisputable indicator of fecal contamination from warm blooded animals. Its thermotolerance facilitates its separation from most other members of the coliform group using selective and differential methods. Replacement of the fecal coliform indicator with *E. coli* has been considered because of thermotolerant-fecal coliforms whose presence in aquatic environments is not necessarily related to fecal contamination. Klebsiella is a ubiquitous FC-positive organism that can be found in aquatic environments and is associated with plant materials (Bagley and Seidler 1977), industrial effluents (Huntley, Jones and Cabelli 1976), and degraded water quality (Vlassoff 1977). The validity of the approved fecal coliform method has been questioned because of FC-positive klebsiellae and estuarine "fecal coliform mimicking" bacteria that proliferate in shellfish harvested from approved growing areas in the Gulf of Mexico and other areas during seasons of maximum water temperature (Miescier et al. 1985). Hood et al. 1983) reported *Klebsiella spp.*were abundant in Gulf of Mexico oysters and clams during the months from April through October. A similar phenomenon may also occur to a lesser extent in the water column. Another criticism focuses on the multiple species composition of the fecal coliform group. It is reasonable to assume that the development of rapid and direct enumeration methodologies could be simplified if the target was a single species. For these and other reasons *E. coli* has been suggested as a more specific indicator of fecal contamination in estuarine waters.

However, we should ask if the information available is sufficiently compelling to support this conclusion. As noted for fecal coliforms, *E. coli* does not fulfil the requirements of an ideal indicator for various reasons despite the alleged exclusivity of its fecal origin. Besides its persistence and aftergrowth capabilities, its biomass is significantly impacted by the natural microbiota. In contrast, we would not anticipate that human enteric viral pathogens would enter the microbial food web and be removed through similar predator-prey or other microbially-mediated interactions. This hypothesis implies a fundamental difference between viral and bacterial indicator removal mechanisms. A selective removal process of this kind may contribute to viral isolations without detecting indicator bacteria. Therefore, although *E. coli* may be a useful indicator downstream from definitive or large, stable point sources of fecal contamination, its value as a quantitative standard in shellfish growing waters impacted by diffuse sources appears equivocal. This conclusion stands despite anticipated approaches to "fine-tune" the specificity, selectivity and accuracy of traditional or 'state-of-the-art" enumeration methods because the deficiencies noted owe their origin to ecological phenomena.

The APHA approved MPN method using lauryl sulphate tryptose or lactose broth followed by EC broth is effective but suffers from the poor analytical precision because it is a statistical estimate of the true population density and is time consuming. The most-probable number based A-1 method was developed by Andrews and Presnell (1972) and subsequently modified as A-1-M (Andrews et al. 1978) as a one step enumeration procedure. The modified method incorporates an initial low temperature (35°C±0.5°) 3 hour resuscitation period followed by incubation at 44.5°C±0.2° in a waterbath for 21±2 hours (APHA 1984). Cook (1981) developed a temperature control mechanism to automatically program an incubator for the required resuscitation and incubation periods at the appropriate temperatures. *E. coli* may be enumerated by direct count using the EPA approved mTEC procedure (USEPA 1985). Water samples are filtered through 0.45µm membrane filters, placed on mTEC medium, incubated for 2 h at 35°C to allow for resuscitation of stressed or injured cells, and incubated at 44.5. After 22h the filter is removed and placed on a pad saturated with urea. Target colonies are urease-positive and turn yellow to yellow-brown.

Nonapproved or Emergent Enumeration Methods for *Escherichia coli*. Rapid methods for detection of *E. coli* in water based on hydrolysis of various fluorogenic or chromogenic conjugated substrates have been developed in recent years and few have been

evaluated in shellfish waters. A review of several methods through 1986 can be found in Hartman et al. (1986). Several assays are based on the ability of *E. coli* to produce catabolic enzymes in response to particular substrates that are conjugated to a fluorogenic or chromogen moiety through a β-D glycosidic linkage. Hydrolysis of the glycosidic bond releases the fluorogen or chromogen which can be detected by fluorescence or spectrophotometry. Kilian and Bülow (1976) evaluated a variety of nitrophenyl-conjugated enzyme substrates for rapid detection of bacterial glycosidases in representative genera including *Escherichia* spp. A widely used fluorogenic substrate is 4-methylumbelliferyl-β-D-glucuronide (MUG) which has been shown to be hydrolyzed by *E. coli* and some *Salmonella* and *Shigella* spp. (Kilian and Bülow 1976). However, Dahlen and Linde (1973) found glucuronidase activity in some*Bacteroides* spp. and *Corynebacterium* spp. Basic assumptions concerning these methods include: (1) that the ability to hydrolyze the substrate is uniformly present in the target species, (2) that this ability can be expressed under a given set of cultural conditions, (3) interferences caused indirectly (overgrowth) or directly (false positives) by the presence of other organisms can be minimized to an acceptable level, and (4) that the sample matrix itself does not autofluorescence or contain enzymes that will hydrolyze the assay substrate. Most reports reveal that only a small proportion of *E. coli* are β-glucuronidase negative and cannot hydrolyze this substrate (Freier and Hartman 1987; Adams et al. 1990). However, reports by Chang et al. (1989) and Lum and Chang (1990) suggest the incidence of glucuronidase-negative strains is much higher than the aforementioned reports suggest and caution against use of MUG hydrolysis as a singular method to detect *E. coli*. MPN, plate, and microtiter based methods employing 4-methylumbelliferyl-β-D-glucuronide (MUG) have been described by (Feng and Hartman 1982). Freier and Hartman (1987) developed two membrane filtration media containing MUG and inhibitors to gram-positive bacteria for simultaneous recovery of both total coliforms and *E. coli* within 24 h. The method, which incorporates a 35°C incubation temperature, does not require a resuscitation step and effectively recovers the target organisms from sewage and surface freshwater. There are no reports of these media applied to shellfish waters. Ley et al. (1988) synthesized the glucuronide, indoxyl-β-D-glucuronide, as a less expensive analog to MUG and chromogenic compounds. Watkins et al. (1988) examined the chromogenic glucuronide BCIG (5-bromo-4-chloro-3-indoxyl-β-D-glucuronide) for the specific, differential identification of *E. coli* from wastewaters and shellfish using direct enumeration methods on agar media. Adams et al. (1990) described a colorimetric method for enumeration of *E. coli* based on hydrolysis of the conjugate p-nitrophenol-β-D-glucuronide in a liquid freshwater medium at 37° and 44°C. The method is based on a graded response whereby the time required for spectrophotometric detection

of the chromogen is inversely related to the *E. coli* cell density. Relationships between log cell density and time to detect dye is established on the basis of a series of standard curves. Interference, demonstrated in the presence of high numbers of competing microorganisms such as *Klebsiella* spp. and *Enterobacter* spp., resulted in overestimations of the "true" cell densities. It is probable that sublethal stress caused by hyperosmolarity, temperature stress, sunlight, starvation, etc. would also cause departures from predicted densities.

The comparative advantages of direct counting methods compared to enumeration methods based on cultivation of target cells are widely recognized (e.g., Daley 1979; Atlas 1982). Specific issues concerning indicator enumeration based on viable recovery methods have been reviewed by Roszak and Colwell (1987). Many studies demonstrating nonculturability have used *E. coli*, *Salmonella* spp. or *Vibrio* spp. as target organisms. A variety of direct counting procedures to distinguish viable from non-viable cells have been developed. Representative approaches include cell elongation (Kogure et al. (1979), measurements of electron transport (Zimmerman et al. 1978), autoradiography (Tabor and Neihof 1982), use fluorescent antibodies (Desmonts et al. 1990), measuring the change in activity over time of ^{3}[H]-thymidine-labeled DNA recovered from cells exposed to seawater (Servais et al. 1985), and recombinant methods such as gene probes and PCR-based amplification of target sequences (Steffan and Atlas, 1988). The APHA recognizes use of fluorescent-antibodies as a valid method for direct detection of microorganisms (APHA 1989). Singh et al. (1989) demonstrated the advantages of combined automated image analysis with the direct viable counting method of Kogure et al. (1979) for bacterial enumeration. Advantages include rapidity and improved counting efficiency. Although this method was used under ideal conditions, e.g., pure cultures of *E. coli* grown in laboratory media, the concentration of nalidixic acid used was observed to significantly affect direct viable counts, the effect generally being to decrease counts with increased antibiotic concentration. Furthermore, the exposure time and concentration of nalidixic acid required to maximize the counts of pure cultures of *E. coli*, *S. typhimurium*, *P. aeruginosa*, *Y. enterocolitica*, and *V. cholerae* varied over almost a ten-fold range. This observation emphasizes the importance of prior knowledge of the effects of nalidixic acid concentration, assay incubation time, and substrate on enumeration of a target indicator or segment of a microbial community in natural samples by direct viable count.

Thus far, methods using recombinant DNA/RNA techniques applied to environmental microbiology have included direct detection through hybridization of the target nucleic acid sequence using naturally occurring organisms in an environmental sample or biological

matrix, or (2) detection by colony hydridization after cultivation of the target microorganism on a particular medium. Although the first method has been limited by amount of DNA or RNA required to hybridize with a probe, use of cell concentration techniques (Somerville et al. 1989) and new PCR methods (Atlas and Bej 1990; Bej et al. 1990) offer improved sensitivity. Atlas and Bej (1990) claim a sensitivity of 1-10 fg of genomic DNA which is equivalent to only 1-5 *E. coli* cells in a 1-100 ml water sample. Issues of concern with the viable approach include those problems associated with viable recovery methods, e.g., interference and overgrowth of the target organisms, poor selectivity and specificity, sublethal stress, nonculturability, etc. Amy and Hiatt (1989) illustrated several of these problems using a DNA chromosomal gene probe against colony blots prepared from water samples containing indigenous microbiota and target *E. coli*. Optimum conditions for detection of target cells were the absence of a competing microbiota and use of selective pressure, conditions that are probably unrealistic with natural samples. Knight et al. (1990) used a commercially available DNA probe for direct detection of *Salmonella* spp. from estuarine water samples Sterivex filter units (Millipore Corporation, Bedford, MA) were used to concentrate and recover cells from water samples. Similar methods could be applied to recovery of indicator bacteria. The probe hybridization procedure, which can be accomplished in 2 days, detected *Salmonella* spp. DNA in direct extracts of cell concentrates and in samples negative for cultured cells following addition of medium to the filter unit . At this time it is not possible to determine with sufficient accuracy the number of cells, viable or nonviable, corresponding to a given yield of probe-bound DNA. Sayler and Layton (1990) recently reviewed environmental applications of nucleic acid probe techniques for detection of microorganisms.

Fecal Streptococci. The term "fecal streptococci" has been used to describe a group of taxonomically diverse streptococci that are Gram-positive, catalase negative, nonspore-forming, facultative anaerobes associated with the gastrointestinal tracts of humans and animals. Functionally, the fecal streptococci were variously divided into groups and subgroups on the basis of serology and physiological characteristics. Thus, the serologically-defined group D streptococci of the fecal streptococci (Clausen, Green, and Litsky 1977), was divided into an "enterococcal" subgroup (*S. faecalis*, *S. faecium*, *S. avium*, and *S. durans*) and a "nonenterococcal" subgroup (*S. bovis* and *S. equinus*). As noted, the later term is curiously inappropriate and has contributed to confusion regarding the meaning of the term "enterococci" (Deibel and Hartman 1984).

Recent studies have indicated that although the "nonenterococcal" species of the genus *Streptococcus* shared the group-D antigen and a fecal habitat, these similarities did not reflect underlying taxonomic relationships. Comparative analysis of oligonucleotides from 16S-rRNA of representative streptococci (Ludwig et al. 1985), and nucleic acid hybridization studies, have led to creation of a new genus, *Enterococcus* (Schleifer and Kilpper-Balz 1984), that includes members of the enterococcal group as well as other species not associated with humans. The genus *Enterococcus* now includes 9 valid species and proposals for the inclusion of 3 newly discovered species have been made (Facklam and Collins 1989). *S. bovis* and *S. equinus*, were taxonomically distinct and not considered valid members of this genus owing to differences in physiology and metabolic characteristics (Bergey's Manual of Determinative Bacteriology 1986).

Although far from being numerically-dominant in human feces, constituting only about 0.1% or less of the gut microbiota (based on cell densities) (Holdeman et al. 1976), this group has been considered as an important indicator of fecal pollution for a considerable period of time. This is attributed to its association with feces, the alleged differences in numbers of coliforms to numbers of fecal streptococci in human and animal feces (Geldreich and Kenner 1969), and observations suggesting that the enterococcal group does not manifest many of the negative characteristics associated with the coliform group, one of the most important being its reported inability to grow in seawater (Slanetz and Bartley 1965). As a group, the complex nutritional requirements of these organisms are generally considered to preclude extraenteral growth (Mundt 1982).

Use of the ratio of fecal coliforms to fecal streptococci has been accorded considerable importance in the literature and much discussion has concerned its validity as an index capable of revealing the nature of a pollution source, i.e., human versus animal fecal contamination (Geldreich and Kenner 1969; Feachem 1975). Values of the ratio equal to 4.0 or more are assumed to reflect human pollution; values less than 4.0 animal (Geldreich and Kenner 1969). This is because there are proportionately more fecal streptococci in the feces of animals than man. A number of investigators have presented data that critically questions the validity of this concept in freshwater environments, including accepted values of the ratio in feces (McFeters et al. 1974; Wheater, Mara, and Oragui 1979). Values of the ratio in feces from a variety of animals and man varied considerably and certain animal species exhibited mean values essentially similar to human sources (Kjellander 1960; Wheater, Mara, and Oragui 1979). Furthermore, the ratio did not remain stable in receiving waters and was difficult to interpret in systems with multiple sources of fecal

pollution. Significantly less attention has focused on the survival characteristics of fecal streptococci in marine and estuarine waters (Slanetz and Bartley 1965; Vasconcelos and Swartz 1976; Lessard and Sieburth 1983). These studies have yielded contradictory results so that a definitive conclusion regarding use of the ratio in these waters is not possible. However, in an estuarine environment, differential dieoff of fecal coliforms and fecal streptococci and the species comprising these groups, and the effects of biological and physical/chemical factors, will likely contribute to variations in the ratio suggesting it would be difficult to deduce the origin of pollution based solely on its value. Furthermore, it is difficult to see how the recommended criteria for use of the fecal coliform/fecal streptococcus ratio (Geldreich and Kenner 1969), no doubt derived with defined point sources in mind, can be met in growing areas polluted by multiple, diffuse sources of fecal contamination. Finally, as will be noted, enumeration of fecal streptococci in estuarine waters is very method sensitive and contributes a source of variation that must be minimized if the ratio is to be viewed as a meaningful parameter (Brodsky and Schiemann 1976). Attention to reduce variability is also important because these organisms occur at significantly lower densities than fecal coliforms. Fortunately, the newest edition of Standard Methods (APHA 1989) cautions against the use of the ratio to differentiate pollution source.

Although certain genera (e.g. *E. faecalis*, *E. faecium*) of the fecal streptococci have been considered specific to feces from human or other warm-blooded animals, phenotypically-similar strains and biotypes can be isolated from other environmental sources (Mundt 1982; Clausen et al. 1977; Beaudoin and Litsky 1981). Strains and biotypes of *E. faecalis* and of *E. faecium* can be isolated from a variety of plant materials, reptiles and insects (Mundt et al. 1958; Mundt 1963; Geldreich et al. 1964; Geldreich and Kenner 1969). Moreover, Mundt et al. (1962) reported growth of *E. faecalis* biotypes on plants and vegetables. In comparison, the distribution and viability of *S. bovis* and *S. equinus* in extraenteral environments appears restricted, exhibiting significantly reduced survival in aquatic habitats (Slanetz and Bartley 1965; Geldreich and Kenner 1969; Wheater et al. 1979). The usefulness of this group as an indicator of fecal pollution in shellfish growing waters appears to depend on development of specific enumeration methods that distinguish between strains of fecal versus non-fecal origin.

A large variety of media have been developed for the recovery of fecal streptococci (Brezenski 1973; Yoshpe-Purer 1989). Methods have been based on most-probable-number techniques, direct pour plate or membrane filtration techniques, some of which

incorporate resuscitative steps to minimize sublethal stress. Comparisons of the various media and methods can be found in references such as Switzer and Evans (1974), Clausen et al. (1977), Pagel and Hardy (1980), Voltera et al. (1985) and Yosphe-Purer (1989). It is evident from this literature there exist considerable differences in recovery efficiency, specificity and selectivity among the different methods, and that each method requires some degree of confirmatory testing. Use of this group as an indicator in marine and estuarine shellfish waters requires improved methods, specifically the selectivity and specificity of media, coupled with an improved understanding of factors affecting the survival of this group. Methods evaluation should be performed in shellfish growing areas, with attention to identifying typical fecal streptococcal species recovered and those microorganisms indigenous to shellfish growing waters that produce false positives. Essentially similar recommendations with respect to methods were articulated quite a few years ago by Kjellander (1960) and Hartman et al. (1966). Thus, until recently KF was considered the medium of choice in the 15th edition of Standard Methods (APHA 1985), although anecodotal reports by many investigators and most recently Yosphe-Purer (1989) suggested that KF medium is not sufficiently selective to apply to marine waters. The 16th edition of Standard Methods (APHA 1989) now recommends use of azide-dextrose broth with confirmation on Pfizer selective enterococcus agar (PSE) for MPN determinations and m Enterococcus agar for membrane filtration assay.

Other considerations aside, use of this group as a fecal indicator necessitates adoption of a common functional definition of fecal streptococci (as with fecal coliforms), that is based on a formalized and unique method for recovery of specific enterococcal and streptococcal species associated with feces. This must be viewed as a challenging but formidable task.

Enterococci. The term "enterococci," with its connotation of fecal origin, is "understood" by some workers to exclude those fecal streptoccocal species (*S. equinus* and *S. bovis*) associated almost exclusively with animal gastrointestinal tracts. This usage is misleading because the term implies streptococci sharing a fecal habitat. Diebel and Hartman (1984) consider the terms group-D streptococci and enterococci the same. Creation of the genus *Enterococcus* spp. (Schliefer and Kilpper-Balz 1984) (whose constituents are "enterococci"?), which contains several non-human species (Facklam and Collins 1989) but excludes *S. equinus* and *S. bovis*, does little to resolve this semantic confusion.

Functionally, the enterococci are defined on the basis of selective recovery obtained using one of several methods developed for this purpose (e.g., Slanetz and Bartley 1965; Isenberg et al. 1970; D'Aoust and Litsky 1975; Levin et al. 1975). The range of species selected varies with each method, some being more selective for the classic "enterococcal" (*E. faecalis*, *E. faecium*) group organisms than others. In reviewing this literature the reader must be wary of the operational nature of the results and current taxonomic relationships.

The most recent use of enterococci in marine waters is by Cabelli et al. (1983). mE medium (as modified by Levin et al. (1975) and Dufour (1980; who substituted indoxyl-β-D-glucoside for esculin in the primary medium) was used in polluted marine and estuarine waters to derive a statistical relationship between indicator density and the incidence of swimming-associated gastrointestinal disease. Cabelli et al. (1983) concluded enterococcal densities were better predictors of health risk than fecal coliforms or *E. coli*. Prospective epidemiological studies to evaluate the enterococci and other indicators as predictors of enteric disease associated with consumption of raw shellfish were recently conducted and the results being evaluated (A. Dufour, USEPA, personnel communication). Enteroccoci are now recommended as the indicators of choice for recreational waters by the Environmental Protection Ageny (1986).

Validation of the enterococci as an indicator of public health risk in shellfish growing areas will require improved recovery methods. The range of selectivity and specificity characteristics of methods for enumeration of enterococci has been mentioned. Current recovery methods require confirmatory testing of presumptive isolates (Ericksen and Dufour 1986), increasing the time and cost of analysis. Although the mE-based method has been applied to marine and estuarine waters (Cabelli et al. 1983), its utility in nonpoint source impacted shellfish growing areas is undetermined. Similarly, enterococcal presence in stormwater runoff (Geldreich et al. 1968; Pagel and Hardy 1980) and differences concerning its persistence in estuarine and marine waters (Lessard and Sieburth 1983; Slanetz and Bartley 1965; Vasconcelos and Swartz 1976) demonstrate a need for distribution and survival studies. Research requirements include : (1) improving methods for recovery of stressed cells, (2) improving specificity for various *Enterococci* spp. and *Streptococci* spp., (3) reducing the incidence of false-positives, which can vary with temperature, season, and geographic region, and (4) minimizing background growth. The relative occurrence and densities of non-fecal biotypes of *E. fecaelis* and *E. faecium* in nonpoint source and stormwater runoff impacted areas should be determined. Rapid

methods for confirmation of selected enterococcal species, based on serological or biochemical characteristics, could significantly improve its candidacy as an indicator in shellfish growing waters. Until recently there have been few reports of direct methods for enumeration of this group or species identification, e.g., fluorescent antibody-based methods (Abshire and Guthrie 1971; Pugsley and Evison 1975). Bosley et al. (1983) describe a useful and rapid (4 hours) identification method to separate enterococci from group D nonenterococci based on hydrolysis of L-pyrrolidonyl-β-naphthamide (PYR). A colony hybridization method, employing oligonucleotide probes synthesized for specific sequences of 23S rRNA of selected enterococci, was used to successfully detect and identify *E. faecalis*, *E. faecium*, and *E. avium* in mixed culture (Betzl et al. 1990).

Alternate Microbiological Indicators for Water

***Bacteroides fragilis* group.** Although the obligate anaerobic bacteria are the dominant constituents of the human fecal microbiota (Moore and Holdeman 1974), attempts to utilize these organisms as indicators of fecal pollution have been limited. This may be attributed to the need for rigorous anaerobic cultural and enumeration procedures and the assumption that anaerobes do not persist under extraenteral conditions.

Bacteroides spp. are nonsporulating obligately anaerobic gram-negative bacteria considered the dominant genus in human feces (Holdeman et al. 1976; Salyers 1984). Like the bifidobacteria, *Bacteroides* spp. is an important candidate indicator of fecal pollution as its major habitat is restricted to the gastrointestinal tracts of humans and warm-blooded animals. As a group, Bacteroides may be more tolerant of oxygen than the bifidobacteria; some *Bacteroides* spp. produce catalase and *B. fragilis* and *B. distasonis* synthesize superoxide dismutase (Salyers 1984).

Until recently, the term *Bacteroides fragilis* group (BFG), was used to describe dominant bacteroides found in the human colon that included *B. fragilis* and its subspecies. The subspecies were subsequently accorded species rank on the basis of DNA homology studies (Salyers 1984) and dominant species now include *B. vulgatus*, *B. fragilis*, *B. thetaiotaomicron*, and *B. distasonis*. The term BFG still appears in the current literature, an although taxonomically incorrect, has utility as a simple phrase referring to the dominant human colonic bacteroides species. *B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis*. are numerically dominant (10^{10} cells/g dry weight feces) and *B. vulgatus* that can constitute

>10% (as cell count) of the culturable fecal microbiota (Holdeman et al. 1976). *B. fragilis*, which is more aerotolerant than *B. vulgatus*, occurs at densities similar to *E. coli*. The observation that *Bacteroides* species other than those found in humans dominate in ruminants (Macy 1981) supports the use of BFG species as a human-specific indicator. Macy (1981) provides a summary of host-related species composition and methods for recovery and cultivation of non-pathogenic *Bacteroides* spp.

Although *Bacteroides* spp. and *B. fragilis*, specifically, are medically significant and can be isolated from a variety of clinical specimens and blood samples (Goldstein and Citron 1988), there is little information describing the occurrence of nonpathogenic bacteroides in natural waters. Post et al. (1967) first suggested use of the BFG group as an indicator of fecal pollution. Allsop and Stickler (1985) evaluated BFG as a fecal indicator based on its presence in sewage, various freshwater, and marine environments. Organisms belonging to this group were readily isolated downstream from waters impacted by sewage. Because *Bacteroides* spp. disappeared faster from these waters than *E. coli*, Allsop and Stickler (1985) concluded the ratio of BFG to *E. coli* counts reflects "aging" of a fecal pollutant and also proximity to the source. The presence of BFG organisms in natural waters was attributed to human fecal pollution because analysis of feces from a variety of domestic animals and feral birds revealed that BFG species occur at much lower densities (i.e., 10^5-10^{10} fold lower) in common domestic farm animals (cattle, horses, pigs, chickens, and sheep)(Allsop and Stickler 1985). Higher densities occur in domestic pets and seagulls but these were still lower than levels in human feces. A variety of obligate anaerobes including *Bacteroides* spp. were detected during the warmer months at a polluted river site (Daily et al. 1981). These observations, admittedly limited, support the potential use of BFG as a human-specific indicator in nonpoint source impacted shellfish growing areas.

General comments made concerning *Bifidobacteria* spp. recovery methods also apply to this group. Perhaps more work on identification methods has occurred with *Bacteroides* spp. owing to its clinical importance as a pathogen and possible involvement in colon cancer. Allsop and Stickler (1984) developed an improved medium for recovery of BFG from natural waters. Penicillin is incorporated into the medium to inhibit clostridia and membrane filtration (0.22 μm) is used for sample concentration. Application of this medium to samples of sewage, and fresh and marine waters revealed interference by obligate and facultative anaerobes. Increasing the concentration of gentamycin improves the selectivity of the medium but decreases recovery efficiency. A resuscitation period is incorporated to minimize the effects of sublethal injury. Serious consideration of the BFG

group requires a detailed assessment of this recovery method in shellfish growing waters, including its selectivity, validation of confirmatory steps required, as well as data describing the effects of season on BFG occurrence, and persistence in estuarine waters.

Anaerobic bacteria vary in their sensitivity toward oxygen (Loesch 1969). Loesch (1969) classified anaerobes into two groups: (1) those that do not exhibit growth on agar media if pO_2 levels exceed 0.5% were called strict anaerobes, and (2) those that could grow at pO_2 levels from 2% through 8% (mean, 3%) were called moderate anaerobes. *B. fragilis* was found among the most oxygen-tolerant of the latter group. Cells exposed to the atmosphere on an agar surface survived for at least 8 hours with only a small reduction in colony counts. This tolerance explains detection of this organism in environmental waters but also suggests that modifications to sample storage and processing protocols that reduce oxygen exposure could improve recovery and enumeration efficiency. Walden and Hentges (1975) confirmed the moderate tolerance of *B. fragilis* to oxygen and demonstrated that oxygen, not Eh, was adverse to anaerobic growth because anaerobes grew well under conditions of positive, intermediate, and negative oxidation-reduction potentials in the absence of oxygen. Onderdonk et al. (1976) confirmed the independence of Eh on growth but observed that under conditions of continuous culture oxygen concentrations from 10 to 100% were bacteriostatic and elicited a decline in metabolic activity.

Chromosomal DNA probes have been developed to speciate *Bacteroides* as an alternative to conventional phenotypic methods. Roberts et al. (1987) and Morotomi et al. (1988) used whole cell dot blot assays that can correctly differentiate *Bacteroides* by species. Kuritza and Salyers (1985) used a cloned DNA probe to enumerate *B. vulgatus* in feces and concentrations measured were similar to viable counts. Although the probe was highly specific for a segment of the *B. vulgatus* genome, its use for enumeration of cells in environmental samples unlikely because of its low sensitivity (ca. 10^7-10^8 cells required). A sample concentration procedure (e.g., Somerville et al. 1989) that would eliminate problems associated with particulates in estuarine water, i.e., clogged filters, and interfering overgrowth by the autochthonous microbiota (Amy and Hiatt 1989) will be needed. However, these probes should be valuable in supplanting or augmenting the specialized and labor-intensive biochemical identification of presumptive isolates. Use of *B. fragilis* bacteriophage as an alternate indicator is discussed in the section on viral indicators.

Bifidobacteria. *Bifidobacterium* spp. are obligately anaerobic, Gram-positive, nonmotile bacteria that possess a characteristic pleomorphic and branching cell morphology. They are potentially important candidate indicators of fecal pollution as the habitat of many species appears restricted to the feces of adult humans and infants and major groups of warm blooded animals (Levin 1977; Cabelli 1978a; Bergey's Manual of Systematic Bacteriology 1986)). In humans, bifidobacteria are a major component of the intestinal microbiota occurring at densities greater than 10^{10} cells/g dry feces and some species may comprise more than 6% of the culturable microbiota (Holdeman et al. 1976). A unique metabolic feature of this genus is that glucose is metabolized exclusively and characteristically through the fructose-6-phosphate shunt (bifid shunt) (Bezkorovainy 1989). The first reaction step is mediated by the enzyme fructose-6-phosphate phosphoenolketolase (F6PPK). This enzyme can be detected using a colorimetric assay and is a reliable phenotypic characteristic of the genus (Scardovi 1981; Bergey's Manual of Systematic Bacteriology 1986). Bifidobacteria can also utilize ammonium as a sole source of nitrogen. The sensitivity of these organisms to oxygen varies with species and strains, the presence of CO_2 allowing some species to tolerate exposure to air for several hours.

A potentially valuable property of this group relates to its ability to differentiate human from animal fecal pollution based on "human" and "animal" strains (Levin 1977). Tanaka and Mutai (1980) found that *B. adolescentis* and *B. longum* accounted for 74% of the bifidobacterial strains isolated from human feces. Levin and Resnick (1981) were unable to isolate bifidobacteria dominant in human feces (i.e., *B. longum* , *B. adolescentis*) from a variety of domestic and wild animals except swine. Mara and Oragui (1983) confirmed the low occurrence of these microorganisms in animal feces and developed a medium based on sorbitol fermentation, which selects for bifidobacteria derived from human sources. The ability of human-specific strains to ferment sorbitol can vary and not all sorbitol-fermenting bifidobacteria are derived from human feces (Bergey's Manual of Systematic Bacteriology 1986). Nevertheless, the possibility of differentiating human from animal fecal pollution should provide an impetus for the evaluation of this anaerobe.

Use of the bifidobacteria as an indicator of fecal contamination in shellfish growing waters has been considered (Cabelli 1978a), but recent studies have focused primarily on its occurrence and recovery from freshwater environments (Mara and Oragui 1983; Carrillo et al. 1985; Munoa and Pares 1988). Information on the distribution of these bacteria, albeit limited to natural and sewage contaminated freshwater, suggests its use as an indicator of

very recent fecal pollution is valid as the organisms appear incapable of extraenteral growth.

Although obligate anaerobes, Gyllenberg et al. (1960) reported bifidobacteria survive as well as *E. coli* in freshwater. In contrast, Levin and Resnick (1981) observed *B. longum* and *B. adolescentis*, exposed in vitro to fresh and marine water samples, are less persistent than *E. coli*. *B. adolescentis* populations decline considerably after 48 hours of in situ exposure in tropical freshwaters (Carrillo et al. 1985). Under these same conditions *E. coli* densities remain constant or increased. Results of an in vitro experiment to compare survival of *B. adolescentis* in fresh estuarine water (Kator and Rhodes 1988) confirmed these observations using samples of Chesapeake Bay water. *B. adolescentis* cells were incubated in flasks containing membrane filtered (0.2 μm) water at 6°C and 25°C. *B. adolescentis* persistence at 6°C was moderately better than *E. coli*, but at higher temperatures was significantly worse. In contrast, Levin and Resnick (1981) observed considerably less persistence of bifidobacteria from sewage exposed in vitro to membrane-filtered seawater (32 psu). Survival was also independent of temperature (at temperatures of 4°, 12° and 20°C), with approximately 15% of the initial density remaining after about 6 h. Individual species and strains varied in survival capability.

The generally low bifidobacterial densities found in receiving waters may be attributed in part to poor recovery owing to oxygen toxicity and sublethal stress, factors that are exacerbated by selective media and harsh recovery methods. Munoa and Pares (1988) demonstrated the inability of *Bifidobacterium* spp. cells to produce colonies on a selective medium was caused by sublethal injury following exposure to seawater. To minimize the effect of sublethal stress on enumeration, a two-layer recovery procedure was designed that incorporated plating and incubation of the sample on resuscitative medium (reinforced clostridial agar). This was followed with an overlay of BIM-25, a selective medium developed to improve the poor selectivity of YN-6 medium with environmental samples (Levin and Resnick 1981). Unfortunately, *B., adolescentis* did not grow as well on BIM-25 as other *Bifidobacterium* spp. (Munoa and Pares 1988). Improving recovery methods for this group of microorganisms, especially human-specific species, should be an objective of future research efforts. Beerens (1990) described a modified Columbia (Pasteur Production, Bioservice, France) agar medium containing propionic acid that is selective and enhances recovery of bifidobacteria. This and other recovery media should be evaluated using resuscitative techniques and membrane filtration because of the need for sample concentration. Despite the recognition that the bifidobacteria are sensitive to

oxygen, use of prereduced media or an oxygen-free environment for recovery manipulations has not been prescribed. Although Levin and Resnick (1981) suggest oxygen toxicity is not a concern with recovery of bifidobacteria, sorbitol-fermenting strains are catalase negative, and data such as those of Munoa and Pares (1988) and Kator and Rhodes (1988) suggest factors that exacerbate sublethal stress should be minimized. Differences in the tolerance of bifidobacterial species or strains to oxygen (Bergey's Manual of Systematic Bacteriology 1986) may target the choice of a indicator bifidobacterial species. The role of particle association in protecting *Bifidobacterium* spp. and other anaerobes from oxygen in the environment is undetermined. In situ exposure studies are needed to measure the persistence and recovery of selected bifidobacterial species in estuarine waters and sediment.

If using indicators to identify sources of fecal pollution to nonpoint impacted estuarine growing areas, the unequivocal fecal origin and limited survival properties of the bifidobacteria are potentially valuable because their detection implies immediate fresh sources. The appearance of bifidobacteria in feeder streams may be used to locate sources of fecal pollution and to differentiate human from nonhuman sources. Although discrimination of vertebrate source will require studies to verify the restricted host range of the sorbitol-fermenting bifidobacteria species composition in feces of domestic animals, humans, and septic tank effluents, the ability to differentiate human from animal pollution could aid management strategies directed toward reduction of pollutant sources. Finally, various bacteriophages, including those lytic to *Bacteroides fragilis* (Jofre et al. 1986; Tartera and Jofre 1987), are being considered as human-specific indicators of fecal pollution. Similarly, if bacteriophages lytic to bifidobacteria occur in feces or sewage, these could be used as human-specific viral indicators of fecal contamination.

Clostridium perfringens. *C. perfringens* is an anaerobic, Gram-positive, spore forming rod whose presence in receiving waters has been linked to contamination by feces and wastewaters (Cabelli 1977a; Bisson and Cabelli 1980). Bisson and Cabelli (1980) developed a membrane filtration method for *C. perfringens* recovery applicable to saline waters based on a highly selective medium (mCP). The composition of this medium was later modified to reduce the concentration of a costly component without compromising its selectivity (Armon and Payment 1988). Sartory (1986) compared recovery of *C. perfringens* on mCP and egg yolk-free tryptose-sulphite-cycloserine (TSC) agar as membrane-based tests. For a variety of sample types, which included polluted river water,

egg yolk-free TSC was found as selective as mCP and more efficient. Egg yolk-free TSC was recommended because of its ease of preparation, lower cost, and availability of a simple confirmation scheme.

Bisson and Cabelli (1980) concluded that *C. perfringens* has value as an indicator of chlorination efficiency and the presence of unchlorinated sources of fecal contamination, and as a "conservation tracer" delineating the areal impact and transport of wastewater effluents. Moreover, detection of vegetative cells in the environment reflects fresh and untreated fecal matter because the persistence of vegetative cells is very short (Bisson and Cabelli 1980). Fujioka and Shizumura (1985) confirmed the utility of *C. perfringens* to detect wastewater discharge into fresh water streams. However, there is doubt *C. perfringens* would be a useful indicator in nonpoint impacted shellfish growing areas because (1) it is so persistent that it may be difficult to index to current pollution conditions, (2) it is widely distributed in soils and sediments, and (3) it is carried into growing areas from extraenteral sources by stormwater runoff and transport of suspended sediment (Matches and Liston 1974, Smith 1975).

Rhodococcus coprophilus. *Rhodococcus coprophilus* is an aerobic nocardioform actinomycete proposed as an indicator of domestic farm animal fecal pollution (Rowbotham and Cross 1977b; Mara and Oragui 1981; Oragui and Mara 1983). As a fecal indicator this organism is unique because it is associated with the feces of domestic grazing farm animals but is not considered an active component of the rumen microbiota (Rowbothan and Cross 1977b). Rowbotham and Cross (1977b) noted that numbers (colony forming units/gram) of *R. coprophilus* in pasture grass and in dung collected from cattle fed this pasture grass were similar, implying no multiplication occurs during passage through the animals. In contrast, significant increases in densities of *R. coprophilus* that occur during incubation of fresh dung at a moderate temperature (ca. 20°C) with water, confirms the coprophilic habitat of this organism. Consequently, this organism is commonly found in pasture grass and soils grazed by cattle and other domestic farm animals. Studies have shown that *R. coprophilus* is absent from human feces but consistently found in feces of cattle, sheep, pigs, horses, donkeys, farm-raised poultry and sporadically in dog and seagull feces (Mara and Oragui 1981).

R. coprophilus can survive both dessication and temperatures of 2°–4°C in dung for periods of up to 6 weeks (Rowbotham and Cross 1977b). Goodfellow and Williams (1983)

concluded that the coccal stage of *R. coprophilus*, carried by runoff into either fresh or salt water habitats, does not grow but remains viable. *R. coprophilus* persists in vitro for 17 weeks in nonfiltered freshwater incubated at 5°, 20° and 30°C, whereas *E. coli* and fecal streptococci disappeared within 5 weeks (Oragui and Mara, 1983). These investigators suggested these and other properties of the organism could be used (in conjunction with other bacterial indicators) to determine the temporal characteristics, location and source of fecal contamination to freshwater systems. Information concerning the distribution and persistence of this organism in estuarine waters are insufficient to evaluate its utility as an indicator in shellfish growing waters. In an experiment using a recovery medium (MM3) devised by Mara and Oragui 1981), Kator and Rhodes (1988) compared its persistence to *E. coli* over a 30 day period in 0.2 μm filtered estuarine water incubated at 6° and 25°C. Although *R. coprophilus* appeared to multiply more slowly than *E. coli*, its persistence in water over the range of salinities and temperatures tested appeared generally better than *E. coli*, and it manifested less initial aftergrowth at 25°C. Whether the observed density increases were due to growth or an artifact of the organism's fragmentable nocardioform morphology (see methodological considerations below) must be addressed in future studies. Similarly, the persistence of this organism in marine and estuarine sediments has not been examined, although it is readily isolated from marine and estuarine sediments (Goodfellow and Williams 1983; Attwell and Colwell 1984; Goodfellow and Haynes 1984; Kator and Rhodes 1989). Isolation of *R. coprophilus* in a small subestuary of the York River, Virginia, was more frequent and the densities higher in water and sediment adjacent to a livestock farm compared to locations lacking this activity (Kator and Rhodes 1989). Studies of *R. coprophilus* persistence in estuarine water and sediment, related particularly to seasonal temperature, are needed to assess its value as an index of current pollution conditions.

Disadvantages now associated with use of this indicator are primarily limitations inherent in the current enumeration procedure (Mara and Oragui 1981). Only small volumes (0.2 ml) of sample can be spread plated onto MM3 agar, currently the medium of choice. Direct spread plating limits detection to 1 cfu/ml (e.g., five replicate plates containing 0.2 ml each). A recovery procedure is needed for concentrating *R. coprophilus* from water using MM3 or other suitable medium for expressing characteristic colony morphology. The combination of membrane filtration (Pisano et al. 1986) with the novel selective method of Hirsch and Christensen (1983) could be evaluated for enumeration of *R. coprophilus*. Hirsh and Christensen (1983) described a "selective" method for recovery of actinomycetes that eliminates bacterial contamination. This is based on the ability of actinomycetes to

penetrate the "pores" of a membrane filter on a growth medium, whereas bacteria remain on the filter surface. After an incubation period the filter is discarded and only actinomycetes that penetrate the filter form colonies. Another limitation noted by Mara and Oragui (1981) is the long incubation period (17–18 days) required for development of characteristic stellate colonies having both substrate hyphae and bright orange central papillae (Rowbotham and Cross 1977a). The prolonged incubation is a consequence of (1) the low organic content of the medium, (2) the presence of inhibitors used to suppress bacterial growth, (3) the photochromogenic nature of color production, and (4) the time required to visibly develop the characteristic colony morphology. Biochemical confirmation of nocardioforms to the generic level is very time-consuming and identification on the basis of colonial morphology alone is neither reliable nor valid (Goodfellow and Williams 1983; Bergey's Manual of Systematic Bacteriology 1986). Rapid and direct methods for species verification (and enumeration) are needed. An indirect method to detect or identify *R. coprophilus* using actinophage appears doubtful because of the broad cross-reactivities of actinophage to both *Nocardia* and *Rhodococcus* (Prauser 1984).

Precise enumeration of *R. coprophilus* is complicated by the inherent morphogenetic nature of nocardioforms, and particularly for *R. coprophilus* , which forms extensively branched hyphae and coccoid elements that are connected in chains of varying length. Thus, a given colony-forming unit in an water or sediment sample can originate from a variety of cell configurations. Rowbotham and Cross (1977a) observed that a typical colony enumerated from dung is derived from single or multiple coccoid elements, the latter being the most prevalent form. The complicating effect of *R. coprophilus* morphology on enumeration is neither a desirable indicator characteristic nor conducive to analytical precision.

Streptococcus bovis. *S. bovis* may be the dominant fecal streptococcus in warm blooded animals (Kjellander 1960; Wheater et al. 1979). Use of *S. bovis* as a specific indicator of animal fecal pollution was first suggested by Cooper and Ramadan (1955). Since their report few investigators have evaluated its use as an indicator of animal fecal pollution in freshwater and none that we know of in marine and estuarine waters. Wheater et al. (1979) claim the proportion of *S. bovis* to total fecal streptococci was largest in ruminants but note the important role of diet and geographic location on this ratio. However, the association of *S. bovis* with ruminants is not unique as it has been be found

in the feces of dogs, cats, various birds, horses and pigs (Kenner et al. 1960; Clausen et al. 1977). Osawa and Mitsuoka (1990) isolated *S. bovis* biotype 1 (mannitol-fermenting) in the feces of koalas on a selective medium. Geldreich and Kenner (1969), Clausen et al. (1977) and Wheater et al. (1979) were unable to isolate *S. bovis* from human feces. Oragui and Mara (1981) drew similar conclusions but did isolate *S. bovis* from sewage effluent. This contradiction was resolved when they located an abattoir that discharging into the sewage treatment facility. However, other workers have isolated *S. bovis* from the feces of healthy humans (Kjellander 1960; Switzer and Evans 1974). Kjellander (1960) isolated *S. bovis* from about 15% of humans examined; Dalton et al. (1986) observed a fecal carriage rate in healthy humans of 10–16%. About 1.0% of the group D streptococci recovered by Abshire (1977) from human feces during an evaluation of a new presumptive medium for this group were *S. bovis*. Osawa and Mitsuoka (1990) indicate mannitol fermenting *S. bovis* (biotype I) are commonly isolated from ruminants whereas mannitol nonfermenting strains (biotype II) are more frequent in human infections. Some workers suggest these contrasting results are due in part to dietary variation and regional effects. Another explanation is that investigators used methods with different recovery efficiencies, selectivity and specificity characteristics. Based on the literature the hypothesis that *S. bovis* is a unique indicator of animal fecal pollution remains equivocal.

Greater concurrence exists concerning the survival of *S. bovis* in natural waters compared with other enteric aerobic cocci. *S. bovis* mortality in freshwater is much greater than *E. faecalis* or *E. coli* (Geldreich and Kenner, 1969; McFeters et al. 1974; Clausen et al. 1977; Wheater et al. 1979). Results of an in vitro exposure experiment using an isolate of *S. bovis*, exposed to filtered feeder stream or estuarine water at 6°C and 25°C, confirmed its inability to persist, especially at the higher temperature (Kator and Rhodes 1988). As stated earlier, poor survival may be viewed as a positive attribute of this indicator because detection of S. *bovis* implies fecal pollution of very recent origin. Conversely, its poor survival violates the requirement that an ideal indicator be at least as persistent as enteric pathogens.

Although *S. bovis* can be isolated with other *Enterococcus* spp. and *Streptococcus* spp. on some of the media used for recovery of these groups (e.g., Switzer and Evans 1974), only one medium is specifically designed for its isolation (Oragui and Mara 1981). Littel and Hartman (1983) described a selective medium for the fecal streptococci that differentiates *S. bovis* from other streptococci and enterococci based on hydrolysis of a fluorogenic substrate and a colorimetric indicator of starch hydrolysis, amylose azure. The ability of *S.*

bovis to ferment starch has been considered an important phenotypic characteristic separating it from *Enterococcus* spp. and other fecal streptococci. Membrane-bovis agar (m-BA) (Oragui and Mara 1981) is a selective medium for *S. bovis* whose specificity is based on the ability of *S. bovis* under anaerobic conditions to utilize NH_4^+ as the sole source of nitrogen and the absence of a requirement for exogenous vitamins. A resuscitation step to minimize the effect of temperature stress is incorporated. m-BA is more specific and more efficient for recovery of S. *bovis* from freshwater and sewage than KF. Subsequently, Oragui and Mara (1984) described a modified m-BA medium (called mm-BA) containing less sodium azide because of reports noting its inhibitory effect on *S. bovis* strains from different geographical regions. Using m-BA Oragui and Mara (1981) reported that more than 65% of the fecal streptococci in animal feces were *S. bovis*. Moreover, a proportion (10%) of typical isolates later confirming as *S. bovis* failed to ferment starch. Isolates lacking this characteristic could lead to underestimation of *S. bovis* densities using the medium of Littel and Hartman (1983). A significant proportion of isolates we confirmed as *S. bovis* that were recovered on mm-BA from freshwater feeder streams and shellfish-growing waters in a subestuary of Chesapeake Bay did not hydrolyze starch (Kator and Rhodes 1991).

Our experience using this medium with samples from freshwater feeder streams and estuarine shellfish growing areas revealed departures from selectivity and specificity characteristics reported by Oragui and Mara (1981; 1984). Thus, in the analysis of approximately 400 mm-BA isolates, a significant proportion (65.1%) of "typical yellow colonies", i.e., presumptive *S. bovis*, were confirmed as *E. faecium*, *S. salivarius* and non-group D streptococci (Kator and Rhodes 1991). This figure is considerably higher than the 1.3% false-positive rate observed by Oragui and Mara (1981). Conversely, in terms of false- negatives 8.9% of the atypical, non-yellow colonies were confirmed as *S. bovis*. *E. faecium* was frequently recovered on m-BA, a point subsequently noted by Mara and Oragui (1984). Although *S. bovis* has potential as a source-specific and time-sensitive indicator, and the mm-BA method has the advantage of sample concentration, use in shellfish waters requires an evaluation of the medium's recovery efficiency, specificity and selectivity over seasons and in different geographic regions. Also needed is development and validation of rapid screening methods for confirmation of presumptive *S. bovis*.

Osawa and Mitsuoka (1990) reported a selective medium for recovery of *S. bovis* based on treatment of brain heart infusion agar with tannin and addition of colistin-oxolinic acid to inhibit enterobacteria in feces. Although the medium selectively recovers and differentiates

S. bovis biotypes I and II present in fresh koala feces, it has not been evaluated with other animal feces, applicability to membrane concentration, and is not particularly effective recovering *S. bovis* in pure culture.

Bacteriophages. Occurrences of enteric viral disease attributed to shellfish consumption (Richards, 1985) have stimulated research for an appropriate viral indicator. Inadequacies of the current bacterial standard have been further emphasized by the lack of parity in survival characteristics of fecal coliforms and enteric viruses (Feachem et al. 1983) and the detection of enterovirus in shellfish from approved growing waters (Goyal et al. 1979; Vaughn et al. 1979; Ellender et al. 1980). Richards (1985) has advocated the direct use of enterovirus as indicators, in particular poliovirus because it is easily cultivated and prevalent in sewage due to universal vaccination. Although cost, method and time constraints currently preclude routine detection of enteric viruses, such analyses could be used to assess potential health risk in growing areas lacking identifiable sources of human fecal pollution and closed to direct harvesting because of elevated indicator densities.

Compared with the costs and limits of direct viral detection, bacteriophage indicators are appealing due to their resistance to disinfection (Keswick et al. 1985) and physical factors that eliminate bacterial indicators (Debartolomeis 1988), their ease of detection, low analytical cost, and short assay periods. Justification for use of coliphages as indicators of fecal and sewage pollution or as simulants of human enteric viruses in estuarine and marine receiving waters (Borrego et al. 1987, 1990; Cornax 1991; O'Keefe and Green 1989; Grabow et al. 1984; Kott et al. 1974; Kott 1981; Scarpino 1975) has been reviewed by Gerba (1987) and IAWPRC Study Group on Health Related Water Microbiology (1991). Gerba (1987) expressed concern over the paucity of basic data describing ratios of specific coliphages to viral pathogens and the occurrence, persistence, and seasonal stability of coliphages in shellfish growing waters. The following studies illustrate the basis of his concern. Vaughn and Metcalf (1975) recovered enterovirus from shellfish growing waters free of coliphages. Seeley and Primrose (1980) described a subpopulation of coliphages apparently capable of replication at temperatures found in freshwater environments. In situ replication of somatic coliphages in estuarine water has been reported (Vaughn and Metcalf, 1975). It is evident that studies of coliphage ecology in marine and estuarine waters are necessary to evaluate its utility as an indicator.

Use of male-specific RNA coliphages as indicators of sewage pollution has been discussed (Havelaar and Hogeboom 1984; Furuse 1987; Havelaar and van Olphen 1989). The gastrointestinal tract of warm blooded animals and domestic sewage are major habitats for these viruses (Furuse et al. 1978). FRNA coliphages share some properties with human enteroviruses such as type of nucleic acid, structure and size, although these similarities do not necessarily translate to similar functional properties in saline environments or shellfish. FRNA phage may also be source specific. Furuse et al. (1978) isolated and classified FRNA phages in domestic waste from various countries in South and East Asia into four major serological groups (groups I, II, III and IV). The relative proportion of FRNA phages in domestic waste and sewage ranges from 10-90% of total coliphages (Furuse et al. 1978; Osawa et al. 1981b). Most FRNA phages from countries other than mainland Japan belonged to group III and those in mainland domestic waste were in group II. Dominance of phage groups in Korea was intermediate between Japan and Southeast Asia, i.e., groups II and III were equally prevalent (Osawa et al. 1981b). Differences in geographic temperature and its effect on viral replication was hypothesized responsible for the distributions observed (Osawa et al. 1981b; Snowdon and Cliver 1969). From a global perspective, fewer FRNA phages were detected in sewage from Central and South American countries than in Asian sources (Furuse et al. 1978). Additional studies (Osawa et al. 1981a) showed FRNA phages belonging to group I were only detected in feces or gastrointestinal contents of mammals (domestic farm and feral zoo animals) other than humans. FRNA phages isolated from pigs belonged to groups I and II, and those from humans groups II and III. Phages belonging to group III were exclusive to humans. However, in a study of domestic sewage from treatment plants in Japan (Furuse et al. 1981), FRNA phages belonging to groups I, II and II were found. Occurrence of group I phages, albeit at low frequencies compared to those in II and III, was attributed to inputs of sewage derived from animal sources such as slaughterhouse wastes. Basic studies concerning the ecology and occurrence of FRNA phages in sewage and feces in the United States are lacking. Poppell (1979) recovered FRNA coliphages at low frequency (2%) from human feces and consistently from all parts of municipal sewage collection systems in the Northeast United States. The validity of FRNA serological groupings to differentiate human and animal fecal contamination should be examined, and, if corroborated, could be useful in nonpoint impacted growing areas.

Two coliphage assay systems have been recently developed that are selective for F-specific phages. Previous coliphage assays required labor intensive screening to identify RNase sensitive phage to avoid counting somatic and filamentous DNA phages. Debartolomeis

(1988) developed and applied a F-specific sensitive host strain to estuarine and marine waters. The assay uses a male *E. coli* host mutant (called Famp) which is also resistant to lysis by DNA somatic phages. RNase has to be incorporated in a parallel assay to distinguish between FRNA and FDNA filamentous coliphages. A second F-specific coliphage assay was developed by Havelaar and Hogeboom (1984) through addition of an *E. coli* plasmid coding for sex pilus production to a F^- *Salmonella typhimurium* host strain (WG49). *S. typhimurium* was chosen as the host to avoid interference by somatic coliphages which are abundant in sewage. Theoretically, plaques observed on this host would be attributed primarily to FRNA phage because somatic salmonellae and FDNA filamentous phages occur at much lower densities in sewage. This assay has been extensively applied to studies of feces and wastewaters (Havelaar et al. 1984; Havelaar and Nieuwstad 1985; Havelaar et al. 1986) but not to marine or estuarine waters. Recently we compared densities of fecal coliforms and phages lytic against F^+ *S. typhimurium* WG49 from samples collected along a salinity gradient in an estuary subject to nonpoint pollution (Rhodes and Kator, in press). Verified FRNA phages were infrequently recovered from 100 ml samples of feeder streams or estuarine water with fecal coliform densities significantly above the growing area standard (range = <2-7900 FC 100 ml^{-1}). Moreover, higher levels of phages were detected in estuarine sediments compared with feeder stream sediments. Although fecal coliform densities in freshwater feeder stream sediments were one to four orders of magnitude larger than phage densities, densities of phages and fecal coliforms in estuarine sediments were similar. This pattern of phage distribution was inconsistent with the fecal coliform data which identified the feeder streams as sources of fecal pollution.

Our experience confirms the critical importance of the bacterial host strain to the coliphage assay (Sinton and Ching 1987; Havelaar and Hogeboom 1983; Seeley and Primrose 1982). Host strains vary as to the accessibility, specificity and location of phage receptor sites. Evaluation of coliphage indicator systems must be conducted and verified using field samples collected to obtain seasonal coverage. Seeley and Primrose (1982) considered coliphages inappropriate indicators because of the likelihood of interfering phages. For example, Vaughn and Metcalf (1975) found the abundance of coliphages in shellfish growing waters varied over a three year period as a function of the *E. coli* host employed. In the study previously mentioned, Rhodes and Kator (in press) randomly picked plaques on WG49 for confirmation as male-specific phages. Of the total number of plaques produced on *S. typhimurium* WG49, 99% (293 of 294) were produced by RNase-resistant phages. Phages purified from these plaques were lytic against the female parent *S.*

typhimurium WG45, were not lytic against a male strain of *E. coli*, and were lytic against environmental *Salmonella* spp. isolates. The susceptibility of the *S. typhimurium* WG49 host strain to MS2 was routinely retested and confirmed. As a result, not only is the sanitary significance of these data is uncertain, but the application of this particular assay host to nonpoint source impacted growing areas may be inappropriate.

If FRNA phages are to be used as fecal indicators more must be known about their ecology and fate. A possible limitation concerns their comparative occurrence in feces and sewage; FRNA phages are an infrequent component of human (and some animal) feces (Havelaar et al. 1986; Furuse 1987; Havelaar 1987) and occur at densities significantly higher in sewage. Their abundance in sewage treatment plants may be the result of extraenteral multiplication at ambient temperatures on hosts that formed pili at temperatures above 30°C (Havelaar et al. 1986; Havelaar 1987; Havelaar and Pot-Hogeboom 1988). However, Poppell (1979) calculated the low occurrence of FRNA phages found in humans was sufficient to account for the densities found in sewage because the numbers of phages contributed by those individuals was very high. Therefore, the extent to which FRNA phages can infect and lyse enteric bacteria in wastewater facilities, septic systems and growing area environments is an issue of concern because such multiplication would alter the ratios of indicator to bacterial and viral pathogens. Thus, although FRNA (and FDNA) phages may serve as indicators of wastewater and sewage contamination, their use as indicators of fecal contamination or a predictor of health risk in shellfish growing waters requires careful analysis. Unless it can be demonstrated that male-specific phages occur at reasonably high densities in septic leachate, these phages may be a poor indicator of human fecal contamination in non-point impacted shellfish growing areas. On the other hand, FRNA phages may have potential use as an indicator of animal contamination because its is significantly more abundant in the feces of certain domestic farm animals (Havelaar and Pot-Hogeboom 1988; Havelaar et al. 1986).

Another issue of concern is the persistence of bacteriophages in estuarine environments and shellfish. Borrego and Romero (1985) found selected coliphages persisted for extended periods, i.e., hundreds of days in sterilized seawater, but their numbers decreased rapidly in natural nonfiltered seawater. Mitchell and Jannasch (1969) reported that filtered natural seawater (using either 0.45 μm or 0.22 μm membrane filters) is strongly antiviral toward coliphage ΦX-174, producing a decrease of viral titer from 10^{12}/ml to 10^{3}/ml in 6 days. To assess the validity of bacteriophages as indicators of viruses, studies are needed to

examine the survival of target phages under various seasonal conditions in natural waters, sediments, and commercial species of shellfish.

Quantitative assessment of F-specific phages in estuarine waters may require processing sample volumes of 100 ml or larger and the use of concentration methods. Cornax et al. (1991) concluded (perhaps prematurely) that male-specific coliphages and *B. fragilis* bacteriophages are inappropriate indicators in seawater because of their low densities in sample volumes of only 0.1 to 1.0 ml assayed by double-layer-agar method. Grabow and Coubrough (1986) used a single-agar-layer method to assay 100 ml sample volumes although this is time consuming and costly. Methods for phage concentration have relied primarily on concentration techniques developed for human enteric viruses. However, there is no a priori basis to assume these methods are appropriate or equally effective for bacteriophages (Borrego et al. 1991). Seeley and Primrose (1982) reviewed a variety of viral concentration procedures and considered their applicability to phage concentration. Positively charged filters have been used successfully to concentrate coliphages from potable and freshwater systems (Logan et al. 1980; Singh and Gerba 1983). Although positively-charged filters are considered effective for concentrating enterovirus from estuarine waters (Kilgen and Cole 1983), Havelaar (1986) reported they were ineffective for recovery of coliphage from artificial seawater. Concentration methods for enteric viruses from marine waters have relied primarily on the use of electronegative microporous filters (Sobsey 1987). Debartolomeis (1988) evaluated a method for recovery and concentration of FRNA bacteriophages modified after the procedure of Purdy et al. (1984, 1985) for recovery of *Bacillus* spp. bacteriophage from water samples. The procedure involves adsorption of bacteriophages to host cells added to a water sample, concentrating the infected cells by centrifugation or membrane filtration, and plaque assay of the concentrate by agar overlay. Debartolomeis (1988) obtained mean recoveries of 103.5 and 87.4 percent, respectively, for naturally-occurring F-specific phages in seawater and river water when compared against the direct overlay method. Using the same approach with marine samples spiked with *Bacillus* spp. phages, Purdy et al. (1984, 1985) obtained anomalous results. Gerba et al. (1978) described a successful protocol for concentration of poliovirus from seawater using pleated membrane filters in conjunction with aluminum chloride flocculation. Isbister et al. (1983) incorporated 2,3,5-triphenyltetrazolium chloride into a single layer coliphage assay procedure to improve plaque visualization. Borrego et al. (1991) evaluated a variety of electronegative and chemically-produced electropositive filters for recovery of coliphages from nonsaline water containing diluted sewage effluent A protracted "drop-by-drop" elution technique with 3% beef extract and positive pressure

gave superior recovery of coliphages adsorbed to diatomaceous earth filters treated with cationic polymers. Application of these and other methods for enumeration of F-specific and other phages requires further evaluation before being considered for routine use in shellfish growing waters.

Sobsey et al. (1990) recently described a simple membrane filter-based method for concentration of bacteriophages from drinking and surface water. This method may have applicability to shellfish waters provided that particulate loading on the filter is not an uncontrolled source of variation to overall recovery. Briefly, $MgCl_2$ is added to a water sample, which is then vacuum filtered through a 0.45 μm membrane filter, and the filter placed face down on an agar surface inoculated with the assay host. Adsorbed phages subsequently desorb to produce plaques in this agar layer and a tetrazolium dye is used to improve detection. Particulates were shown to reduce plaque numbers, possibly by competitively inhibiting phage adsorption, suggesting this method will have to be carefully evaluated in estuarine waters which are generally characterized by high particulate loads. Methods for the recovery of coliphages from a variety of representative estuarine and marine sediments must be developed and rigorously verified. In a study of this type, Armon and Cabelli (1988) compared the effectiveness of various eluants to release f2 phage experimentally adsorbed to purified clay minerals and those in natural sediment. Efficient recovery of phages from shellfish will be needed because the numbers are likely to be low because only small volumes of homogenized samples can be accommodated by direct plating methods. Brodisch et al (1986) enumerated coliphages in mussel homogenates by direct plaque assay in a medium augmented by antibiotics to suppress background. A similar approach should be evaluated for different species of commercial shellfish in this country.

Use of direct pour plate assay methods for phage enumeration generally requires steps to reduce or suppress the growth of background microbiota, depending on sample type. Incorporation of antibiotics, decontamination with chloroform (Tartera and Jofre 1987; Osawa et al. 1981a), membrane filtration (Tartera and Jofre 1987), and selective media (Kennedy et al. 1985) have been used for this purpose. Adams (1959) used chloroform to lyse background cells to optimize coliphage recovery. Osawa et al.(1981a) diluted sewage samples with 5 ml of PG medium and treated with chloroform (5%, v/v) to kill bacteria. Kennedy et al. (1985) compared chloroform (5%, v/v) with other methods including selective media to reduce background for recovery of coliphages from sewage and lake waters. Following an exposure interval after mixing of 1 hour to chloroform, samples

were removed from the aqueous phase for phage enumeration. Chloroform eliminated the background but also reduced plaque numbers on most media examined and use of selective media was recommended. Tartera and Jofre (1987) found that although chloroform did not inactivate *B. fragilis* phages, it also did not suppress growth of anaerobic sporeformers, which sporulated under anaerobic conditions. Potassium sorbate (0.05%) and a lowered pH (5.7) were used to eliminate spore germination. Cornax et al. (1990) compared use of chloroform (20%, v/v), antibiotics, and membrane filtration for removal of the background during recovery of *B. fragilis* bacteriophages from sewage and sewage-contaminated seawater. Membrane filters (0.45 μm and 0.22 pore nitrocellulose) were used either untreated or treated with 10 ml of 3% beef extract (pH 9.5). Recovery was statistically better in terms of phage numbers and suppression of background growth using 0.45 μm filters treated with beef extract. It was hypothesized that treatment with beef extract combined with the larger pore size membranes is the most effective because most bacterial cells were retained and yet phages were able to pass unadsorbed through the nonbinding filter matrix. The combined use of membrane filtration with incorporation of antibiotics was recommended to increase overall assay selectivity.

In contrast to coliphage, bacteriophages active against the anaerobe *Bacteroides fragilis* demonstrate a high degree of host strain specificity and appear to lack activity against other species of *Bacteroides* spp. (Booth et al. 1979; Cooper et al. 1984; Keller and Traub 1974; Kory and Both 1986; Tartera and Jofre 1987; Tartera et al. 1989). As a potential indicator of fecal contamination, *B. fragilis* phages were detected exclusively in human feces and sewage and appear to reflect the dominance of the *Bacteroides fragilis* host in human feces (Booth et al. 1979; Cooper et al. 1984; Salyers 1984; Tartera and Jofre 1987; Tartera et al. 1989). Phages lytic to the most efficient host strain examined, *B. fragilis* HSP40, were recovered only from environmental areas subjected to sewage and never detected in nonpolluted areas or those occupied exclusively by feral animals (Jofre et al. 1986; Tartera and Jofre 1987). These observations, coupled with the apparent inability of *B. fragilis* phage to multiply in freshwater, seawater or sediment habitats (Jofre et al. 1986; Tartera et al. 1989), suggest this phage is a promising subject for verification as an indicator of human fecal pollution. The low isolation frequency of HSP40 bacteriophages in humans, i.e., ≤10%, suggests the need for sample concentration and an assessment of its occurrence in waters and shellfish in growing areas contaminated by nonpoint pollution.

The occurrence of phages active against alternate bacterial indicators such as *B. adolescentis* or *B. breve*, *S. bovis*, and *R. coprophilus* appears to have received received

scant attention. Phage assay systems could provide an alternative detection method to viable enumeration procedures which are unsatisfactory for reasons previously discussed. Enumeration of phages active against human specific sorbitol-fermenting bifidobacteria could provide an assay system similar to that proposed for *B. fragilis* (Jofre et al. 1986). As noted, cross-reactivities between *Nocardia* spp. and *Rhodococcus* spp. phages may preclude development of a *Rhodococcus*-assay system.

Although validation of a viral indicator will depend upon establishing a statistically significant relationship between densities of a given phage and target enteric pathogen or risk of enteric disease, such information will at first be very difficult and expensive to obtain. Therefore, candidate viral phage indicators must be carefully evaluated before these studies are begun to understand their ecology and to confirm their target specificities with samples collected from regionally-characteristic growing areas over a variety of seasonal conditions.

Enumeration of Indicators in Shellfish

The object of this section is not an exhaustive coverage of a very large and (sometimes redundant) literature concerning methods of recovery of indicators from shellfish. Rather, its purpose is to highlight areas we believe warrant attention or are of current interest. Specifically, all methods should be considered with regard to factors that affect indicator recovery discussed in previous sections and the overall need to improve analytical precision.

Methods for Shellfish Preparation. Procedures for preparation of shellfish for enumeration of microorganisms vary with the microorganism being sought and the detection method. The first step of the currently approved method (APHA 1985) for detection of coliform organisms is an initial breakup and dilution of a sample consisting of 10–12 whole animals to release and uniformly distribute microorganisms throughout a homogeneous suspension. However, Al-Jebouri and Trollope (1981) found processing only parts of the digestive system (through excision of the "stomach and intestine") of mussels yielded improved sensitivity of bacterial numbers compared with the total animal homogenate method. In contrast, Metcalf et al. (1980) recovered more virus from whole shellfish than from selected tissues which included hepatopancreas from oysters and

hepatopancreas and siphons for clams. Mechanical breakup of the tissue is normally performed by homogenization (low-speed blending or high-speed homogenization) or stomaching. Stomaching, which seems to be preferred for many foods, has only been evaluated for shellfish by a few investigators and it has not been rigorously tested. Trollope (1984) compared counts of lactose-fermenting bacteria from mussels (*Mytilus edulis*) after stomaching and mechanical blending. Average counts from stomached tissue were about 2x higher than mechanically blended tissue. Andrews et al. (1978), in a comparison of stomaching and blending using a variety of foods, found stomaching to recover higher aerobic plate counts (APC) from oysters. A smaller volume model was judged more efficient than a larger one for recovery of APC in oysters. Stomaching is intrinsically a uniform and "clean" method because the sample is encased in a plastic bag, preventing aerosolization and heating because the sample is repetitively struck with a paddle. However, its effectiveness for release of naturally-polluting bacteria and viruses from different species of edible shellfish requires evaluation. It has been claimed for example, that one problem with stomaching shellfish is bag puncturing because shucked shellfish occasionally contain large bits of shell debris (A. P. Dufour, personal communication).

Because of better precision, methods that utilize membrane filtration for direct estimates of target indicators in shellfish may be preferable to MPN-based or direct pour-plate methods. Direct pour-plate methods are limited by small plant volume, the potential for heat-shocking stressed cells, and background interference. The potential accuracy of direct counting methods using membrane filtration for epifluorescence microscopy or culturable counts is influenced by requirements to: (1) optimize release microorganisms from the food matrix, (2) minimize clogging of the filter by reducing the sizes of food particles, and (3) to prevent adsorption of food on and within the filter. Food particles that remain on the filter are a potential substrate for competing organisms and may obscure colony development of target microorganisms. An approach to achieve these goals is to treat shellfish with hydrolytic enzymes. Hydrolysis must breakdown the food matrix without reducing the target indicator population, especially if sublethally stressed cells are present. Trypsin and other enzymes have been used to effectively digest a variety of food types (Entis et al. 1982) for microbiological analysis using the hydrophobic grid membrane filter (0.45μm) method. Enzyme digestion has also been coupled with the direct epifluorescent filter technique (DEFT) for estimation of total microbial numbers in meat and poultry (Shaw et al. 1987). Rodrigues and Kroll (1988) proposed use of DEFT for rapid enumeration of coliforms by counting microcolonies growing on selective media. DEFT methods are amenable to

automated counting by image analysis and use of fluorescent antibodies for added specificity. Although homogenized clams and oysters have been treated with trypsin for enumeration of *E. coli* and enterococci by membrane filtration (A. P. Dufour, personal communication), an evaluation of the usefulness of enzymatic digestion for the microbiological analysis of shellfish is needed.

Whatever methods used for release of microorganisms, different species of shellfish are unique in terms of their overall structure and organization, physiology, and accumulation strategies. Given the same natural exposure conditions, Manila clams (*Tapes japonica*) consistently accumulate coliforms and fecal coliforms at higher concentrations than oysters (*Crassostrea gigas*) (Vasconcelos et al. (1969). Viral infection experiments with *Crassostrea gigas* suggest viruses are absorbed intracellularly, reflecting a different mechanism of sequestering than indicator bacteria (Hay and Scott 1986). Power and Collins (1990) found similar uptake patterns of *E. coli* and an iscohedral coliphage in various tissues of the mussel *Mytilus edulis*. Oysters are considered more easily homogenized than hard clams. Metcalf et al. (1980) observed significant differences in the recovery efficiency of spiked viruses as a function of shellfish species. Use of identical procedures for hard and soft-shelled clams resulted in lower viral recoveries from the latter. Additional extraction steps were necessary to release virus from soft-shell clams. In view of the national interest in alternate indicators and direct counting methods it seems prudent to optimize recovery methods as a function of target indicator *and* shellfish species. Commercially important filter-feeding shellfish include oysters (*Crassostrea virginica, Crassostrea gigas, Crassostrea lurida*), hard-shell clams (*Mercenaria mercenaria, Mercenaria campechiensis*), soft-shell clams (*Mya arenaria*), Pacific little neck clam (*Tapes japonica*), and mussels (*Mytulis edulis*).

Approved Methods for Indicators

Total coliforms and fecal coliforms. One approved method for examining the bacterial quality of shellfish is the multiple tube fermentation test (APHA 1985). Used in either 3- or 5-tube most-probable-number (MPN) configurations, the method offers poor precision, providing an estimate of the "true" population and large confidence intervals. The largest sample portion generally inoculated is 10 ml of a 1:10 diluted homogenate which is equal to 1 gram of shellfish meats. Media used are the same as for water, lactose

or lauryl sulfate tryptose broths for the presumptive test, followed by EC broth and brilliant green bile broth (BGB). Total analytical time for presumptive and confirmed tests is 72 hours for fecal and 96 hours for total coliforms. Confirmation that fecal coliforms are *E. coli* with this method takes 10 days. The validity of the approved fecal coliform MPN test, and particularly its use to assess product quality in terms of the recommended market guideline of 230 FC/100 grams meats, has been questioned for shellfish from the Gulf of Mexico. Counts exceeding this guideline were attributed to EC-positive klebsiellae and estuarine "fecal coliform mimicking" bacteria which proliferate during seasons of maximum water temperature (Miescier et al. 1985). Hood et al. (1983) reported *Klebsiella spp.* were abundant in Gulf of Mexico oysters and clams during the months from April through October.

There is only one approved pour plate method to detect and measure densities of fecal or elevated temperature coliforms in shellfish (hard and soft clams) meats (APHA 1985). It offers a short analysis time (24 hours) compared with the MPN, the improved precision of a direct count, but is restricted to the shellfish mentioned and less sensitive in that only 1 gram of shellfish meats can be processed per plate. In addition to the important issue of recovery of sublethally-stressed cells (Yoovidhya and Fleet 1981), other problems associated with this method are reports of significant interferences caused by background growth with shellfish from the Gulf of Mexico and variations in dye lot (John Miescer, FDA, personal communication).

Nonapproved and Emergent Methods

A succinct discussion of rapid methods, media evaluated, and international methods for recovery of fecal coliforms and *E. coli* from shellfish meats, is available in the Compendium of Methods for the Microbiological Examination of Foods (APHA 1984). The following sections focus on recent methods not covered in the Compendium.

Coliforms. Detection methods based on hydrophobic grid-membrane filters offer significant advantages in enumeration of indicators in foods (Sharpe et al. 1979). These include larger numerical operating range, improved precision, sensitivity, reduced background interference, and removal of inhibitory substances and substrates that may support growth of background species. These are very desirable characteristics and in

combination with appropriate media could prove valuable with shellfish if a reliable and reproducible method can be developed for the initial filtration step. Entis and Boleszczuk (1990) evaluated a hydrophobic grid membrane filter method for enumeration of total coliforms and *E. coli* within 24 hours. The method compared favorably with a MUG-based 3-tube MPN. The method has been used for scallops and shrimp, digested with trypsin, but not bivalve shellfish.

Escherichia coli. As noted previously the fluorogen, 4-methylumbelliferyl-β-glucuronide (MUG), which has found application for enumeration of *E. coli* in water, has also been examined for similar use in foods and seafoods. The initial work of Feng and Hartman (1982) was followed by Alvarez (1984) and Moberg (1985). Moberg (1985) examined factors such as specificity, sensitivity, and optimum MUG concentration in LST (lauryl sulfate tryptose broth)-MUG to detect *E. coli* in a variety of food and dairy samples. The LST-MUG method had a lower false-positive rate and detected more *E.coli* in non-seafoods than the standard APHA method. False-positives were attributed primarily to staphylococci. Coliforms such as *E. cloacae* and *K. pneumoniae* did not produce detectable fluorescence from MUG in LST. Alvarez (1984) compared recovery of *E. coli* from a variety of fresh and frozen seafoods (including bivalve shellfish). MUG was used directly in lactose broth, in Violet Red Bile agar (VRBA) as an overlay, and in M-Endo broth with membrane filters. All assays were performed within 24 hours. The high specificity of MUG for *E. coli* was verified by confirming fluorescent tubes or colonies in EC broth, streaking on EMB, and with traditional confirmatory tests. The lowest false-positive rates (EC positive, no fluorescence) and highest recoveries were found using lactose broth-MUG. This latter observation was attributed to the nonselectivity of lactose broth toward stressed cells, an observation mirroring earlier findings of Feng and Hartman (1982) who recovered heat-stressed cells on LST-MUG. Koburger and Miller (1985) applied MUG-LST to enumeration of *E. coli* in oysters. They found incorporation of MUG into LST was impractical because oysters possess endogenous glucuronidase activity which yields false positives. Incorporation of MUG into EC broth eliminated this problem although an additional 24-48 h is required. The 25 oyster samples tested yielded 127 gas-positive EC tubes. Of this total 103 tubes were fluorescence-positive and only one was not positive for *E. coli*. Twenty-four tubes were both fluorescent-negative and *E. coli*-negative. However, Chang et al. (1989) suggest that the incidence of glucuronidase-negative strains of *E. coli* is is higher than commonly observed and may lead to underestimations of *E. coli* densities in shellfish or water samples. Frampton et al. (1988)

evaluated another glucuronidase substrate, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-GLUC) as an alternative to MUG. This chromogenic substrate does not require illumination by near-UV to detect β-glucuronidase activity and does not diffuse into agar (as MUG) but remains within a colony. Disadvantages includes high cost, poor intensity in liquid medium, and possible interference if additional color-based tests are performed. Finally, as previously mentioned *E. coli* has been enumerated from shellfish by combining trypsin digestion with membrane filtration and incubation on mTEC (A. P. Dufour, personal communication). Because of the advantages of direct count, this method should be evaluated with regard to reducing the potential deleterious effects of hydrolysis on recovery of stressed cells.

Recovery of other indicators such as the enterococci and bacteriophages will require development and validation of rapid and accurate methods. Methods currently available for recovery of bacteriophages from shellfish meats are tedious, with recovery efficiencies that may vary with species of shellfish. Direct plating of homogenates using the conventional double-layer-overlay procedure lacks sensitivity and is visually demanding to read. Clearly, methods for recovery of viral indicators from shellfish meats must be improved in sensitivity, reliability, and ease of use if they are to become routine.

CHEMICAL INDICATORS

Coprostanol

Coprostanol (5β(H)-cholestan-3β-ol) is formed by bacterial reduction of cholesterol and is one of the principal sterols in the feces of man and other mammals (Murtaugh and Bunch 1967; Walker et al. 1982). Unique anaerobic cholesterol-reducing bacteria, possibly *Eubacterium* spp., use cholesterol as an electron acceptor, reducing the 5,6-double bond of the molecule to form coprostanol (Sadzikowski et al. 1977). Individuals vary as to the amount of coprostanol produced. Wilkins and Hackman (1974) reported that 25% of a human test group reduced less than 50% of fecal cholesterol to coprostanol. Coprostanol can be the dominant sterol in domestic wastes.

Unlike microbial indicators which may be subject to chemical, biological, and physical processes that influence their numbers and detection, coprostanol offers advantages of an abiotic marker of sewage pollution. The areal distribution of coprostanol has been used to

delineate impacts of point sources such as sewage outfalls or dumping areas (Kanazawa and Teshima 1978; Hatcher et al. 1981; Boehm 1983; Pierce and Brown 1984; Brown and Wade 1984; Düreth et al. 1986; Holm and Windsor 1986) but has not been applied to the study of nonpoint pollution. Attempts to correlate densities of bacterial indicators to coprostanol concentrations in estuaries subject to known inputs have yielded conflicting results (Goodfellow et al. 1977; Churchland and Kan 1982; and Yde et al. 1982). Dutka et al. (1988) applying a "battery of biochemical, microbiological and bioassay tests" to evaluate the water quality of the Saint John River and its basin, concluded that fecal sterols were not a useful indicator of fecal pollution.

Concentrations of coprostanol in estuarine (Holm and Windsor 1986) and marine (Kanazawa and Teshima 1978) waters decrease with increasing distance from sources. Brown and Wade (1984) demonstrated that coprostanol in sewage effluent is primarily associated with particulate matter, which may be deposited immediately proximate to an outfall or transported out of the area depending on the dynamics affecting the distribution of fine-grained sediments. Increases of coprostanol concentrations with depth in estuarine waters is attributed to settling of sewage-associated particulates and resuspension (Wade et al. 1983). The distribution of coprostanol concentrations in waters adjacent to the entrances of major estuaries from small- and large-scale physical processes and the buoyancy characteristics of particulate matter. The hydrophobic nature of coprostanol causes it to be associated with particles whose transport subsequently affects its spatial distribution.

Serious consideration of coprostanol as an indicator in nonpoint impacted shellfish growing areas remains hampered by the lack of data describing background levels in such environments and possible origins from nonfecal sources. Laboratory studies using estuarine water and sediment amended with radiolabeled cholesterol demonstrate its conversion to coprostanol by microorganisms (Teshima and Kanazawa 1978). Nishimura and Koyama (1977) suggested that stanols in recent sediments are derived from phytoplankton and sterol conversion. Under anaerobic conditions 5β-isomers of stanols are produced from autochthonous sedimentary organic matter (Nishimura 1982). Tornabene et al. (1974), in a report describing sterols of the diatom, *Nitzschia alba*, noted cholesterol has been identified in both blue-green and red algae. Cholesterol and other sterols have been detected in both surface waters (Matthews and Smith 1968) and sediments (Attaway and Parker 1970) from the Gulf of Mexico. Kanazawa and Teshima (1971) found cholesterol was usually the dominant sterol in both suspended and dissolved

fractions in Kagoshima Bay, Japan. Pocklington et al. (1987) concluded that coprostanol was an equivocal indicator of fecal pollution, identifying the marine biota as a probable source because patterns of coprostanol occurrence in particulate matter were conjunctive with those of various chemical indicators of primary production, including natural phytosterols.

Qualitative and quantitative aspects of coprostanol degradation in estuarine systems remain poorly understood. Coprostanol appears to persist, particularly under anaerobic conditions, in both lacustrine (Nishimura and Koyama 1977) and marine (Bartlett 1987) sediments. Coprostanol attributed to marine mammals (Venkatesan et al. 1986) dating to 3500 B.C. has been found in Antarctic sediments remote from anthropogenic inputs. Recovery of 5β-stanols from late Pleistocene sediments has also been reported (Nishimura 1982).

Observations that demonstrate the longevity of coprostanol as a geochemical marker raise questions concerning its applicability as a quantitative indicator of health risk or as a marker for growing areas affected by varied and intermittent pollution sources such storm or agricultural runoff. Eganhouse et al. (1988) noted concentrations of coprostanol and linear alkyl benzenes (the anionic surfactants found in household laundry detergents)) were rather variable in samples of sewage sludge. This was attributed to differences in concentrations of coprostanol in human feces and the differential effects of biosynthetic and degradative processes that can take place in feces and during waste processing. Another argument against the use of coprostanol is that although samples can be preserved for analysis at a late date, the analysis remains technically detailed, lengthy, and costly. Alternate approaches may result in shortened processing time. Wun et al. (1979) described a column adsorption method using XAD-1 resin (Rohm and Haas, Inc., Philadelphia) for the rapid extraction of both cholesterol and chlorophyll a from water samples. Hoskin and Bandler (1987) described a rapid thin layer chromatographic method for detection of fecal contamination through recovery of coprostanol from foods. Although this method would be of limited value for analysis of water samples, it could possibly be adapted for use with shellfish meats. Current improvements in solid phase packings, especially the silica-based bonded packings, could result in rapid and improved recovery procedures for detection of cholesterol from shellfish waters or shellfish. Krahn et al. (1989) described a rapid, semi-automated high performance liquid chromatographic (HPLC) method for separating coprostanol from interfering compounds in sediment extracts. Finally, Eganhouse et al. (1988) sounded a cautionary note concerning prior and future quantitative surveys of

coprostanol; unless its co-eluting , epicoprostanol, is clearly resolved, reported concentrations have been and will be overestimated. Overall, the preponderance of information suggests the use of coprostanol as an indicator of fecal pollution in shellfish harvesting waters should be viewed as very low priority, because of multiple sources and absence of data supporting its use to nonpoint polluted areas.

Hydrolytic Enzymes

Measuring the activities of selected hydrolytic enzymes as indicators of fecal pollution has been proposed. Lenhard (1967; 1969) examined the relationship between urease activity and the presence of sewage or sewage contamination in bottom sediments. Skiba and Wainright (1982) evaluated urease activity in unpolluted and sewage-polluted beach sands as an indicator of sewage contamination. Urease activity was relatively high in sands adjacent to a sewage outfall and decreased with distance from the source. Urea is a major nitrogenous compound in urine, untreated or partially treated sewage, and its release presumably elicits elevated urease activity in impacted sediments owing to its availability as a bacterial substrate. Urease may also be present in the sewage source. Urease activity can quantified by measuring evolved NH_4^+ (Lenhard 1969) or loss of urea (Sadler 1989). Either procedure is relatively uncomplicated and rapid compared with the standard fecal coliform assay. Positive relationships between urease activity associated with known fecal contamination (Lenhard 1967) and *E. coli* densities (Sadler 1989) have been observed but the data base is very limited. Because the urease assay measures a soluble constituent, it is probable that its ability to reflect the behavior of free- or particle-associated bacterial or viral pathogens in estuarine waters would be imperfect. Data are also needed that describe how quickly sediment urease levels change in response to changing levels of sewage contamination. Compared with coprostanol urease might be a simpler and less labor intensive alternative to trace established sewage plumes or discharges.

Long-Chain Alkylbenzenes, Fluorescent Whitening Agents and Sodium Tripolyphosphate

Certain chemicals found in commercial and domestic detergents and present in municipal wastewaters and domestic sewage have been identified as waste-specific markers or indicators of sewage contamination. Eganhouse (1986) reviewed the chemistry,

occurrence and fate of long-chain alkylbenzenes in various environments. Because of their chemical stability, he concluded use of these compounds would be to delimit areal and temporal impacts of plumes reflecting discharge of municipal sewage. Close et al. (1989) detected septic contamination of groundwater by measuring fluorescent whitening agents and sodium tripolyphosphate. These compounds, which are present in domestic detergents, were detected in 17% of groundwater samples and significantly correlated with densities of fecal coliforms. The value of these indicator compounds to detect the presence of malfunctioning septic systems in a watershed or transport of septic effluents through subsurface infiltration to estuarine systems has not been evaluated. This will also require determination of compound stability and extent of degradation by microorganisms in natural systems.

PROTOZOA

Acanthamoeba

Free-living protozoans belonging to *Acanthamoeba*, a genus ubiquitous in soils and freshwater environments, have been detected in sewage contaminated sediments from freshwater, estuarine and oceanic dump sites and outfalls (Sawyer et al. 1977; Sawyer 1980; Daggett et al 1982; O'Malley et al. 1982; Sawyer et al. 1987). Generally, the occurrence of *Acanthamoeba* spp. has been associated with elevated levels of fecal bacteria although sediments positive for *Acanthamoeba* spp., enteroviruses, coprostanol, PCBs, and heavy metals were sometimes negative for bacterial indicators (Sawyer et al. 1987). Sawyer et al. (1987) proposed Acanthamoeba as as a monitoring indicator based on persistence of its cysts (for periods up to 2.5 years), the pathogenicity of some strains, and its association with sewage. These amoebae are widely distributed in nature. The significance of their association with fecally polluted marine samples requires further study to determine if in situ *Acanthamoeba* densities reflect utilization of bacteria growing in dumpsite sewage sludge or the direct input of populations carried in the sludge (Sawyer et al. 1982; Daggett et al. 1982). Perhaps, the most likely answer will be a combination of both processes. Quantitative data regarding *Acanthamoeba* densities in human feces, sewage, agricultural runoff and nonpoint impacted areas would be needed to assess its applicability as an indicator in shellfish waters. The complexity and cost of this undertaking, drawbacks associated with indicator organisms that form resistant cysts, the lengthy incubation periods, and degree of expertise necessary to distinguish *Acanthamoeba*

spp. from other genera of free-living amoebae are concerns that do not supports its use as a broadly based indicator of fecal pollution.

CONCLUDING COMMENTS

Some investigators (Berg 1978; Richards 1985)) have proposed direct detection of microbial pathogens as an alternative to indicators, citing documented examples of instances where current indicators failed to predict pathogen presence or pathogens were detected in the absence of indicators. In our view, arguments to retain surrogate microbiological indicators (Cabelli 1977b; James 1979) remain valid because of the unpredictable occurrence of pathogens, variations in pathogen virulence, morbidity rates, and minimum infective dosages within a target population. Ultimately, of course pathogens intrinsically lack the ability to predict risk. Arguments based on the lack of accepted methods for direct and accurate routine detection of pathogens are less compelling. Recent advances in biotechnology suggest the feasibility of direct pathogen detection is now reality. Experimental gene probes for hepatitis A virus (HAV) have been used to detect HAV in polluted estuarine waters (Jiang et al. 1987; Metcalf and Jiang 1988) and a gene probe for Norwalk agent is now being developed. A gene probe for Salmonella spp. (GENE-TRAK Systems, 31 New York Avenue, Framingham, MA 01701) is commercially available.

Emergent methods may revolutionize some aspects of microbiology that traditionally have been associated with frustrating drudgery, elaborate and repetitive procedures, i.e, enumeration and phenotypic characterization of isolates. After more than half a century using essentially unchanged cultivation methods for recovery of the coliform indicator group, new methods for rapid detection are becoming available. These may be based on traditional cultivation methods, direct enumeration procedures, target phenotypic characteristics such as constituitive enzymes or antigenic factors, or use recombinant DNA/RNA technology such as PCR to target an oligonucleotide sequence with high specificity. We welcome these new methods, anticipating their promise of ultimate specificity and rapidity but feel compelled to offer the following cautionary remarks.

First, the best detection systems will not eliminate the need to understand the ecology of an indicator; its survival characteristics and the relationship between environmental exposure and the assay. For example, allochthonous bacteria exposed to marine and estuarine

environments lose their ability to grow on selective and conventional bacteriological media (Barcina et al. 1989; Roszak and Colwell 1987; Rhodes et al. 1983) and important diagnostic characteristics may be altered or lost. False-negatives or misidentification can be a consequence of enumerative or identification schemes based on expression of a sensitive phenotypic characteristic. *E. coli* loses its ability to ferment lactose (Kasweck and Fliermans 1978), β-galactosidase activity was reduced in cells exposed to seawater (Anderson et al. 1979; Munro et al. 1987). Loss or alteration of plasmids encoding for antibiotic and heavy metal resistance occurs in various *Enterobacteriaceae* during long-term starvation (Chai 1983; Caldwell et al. 1989) and streptococcal (and enterococcal) isolates from animal and environmental sources do not consistently yield valid reactions in miniaturized biochemical testing systems (Molitoris et al. 1985; Rhodes and Kator unpublished results). Chang et al. (1989) reported a significant proportion of *E. coli* isolated from human fecal samples were β-D-glucuronidase negative.

Finally, it is conceivable that advances in sensitivity and accuracy of analytical methods could lead to requests for growing area closures based on improved detection of pathogens without considering issues such as pathogen infectivity, host susceptibility, historical incidence of disease, and sanitary surveys. Such action could produce an untenable situation for the shellfish industry and regulatory agencies. Detection of pathogens in approved shellfish growing waters using new (or old) methods should not be construed as prima-facie evidence of health risk. Cabelli's (1978b; 1979) arguments that standards must be based on functional relationships, i.e., correlating rates of illness from prospective epidemiologic investigations with indicator densities, must be heeded.

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